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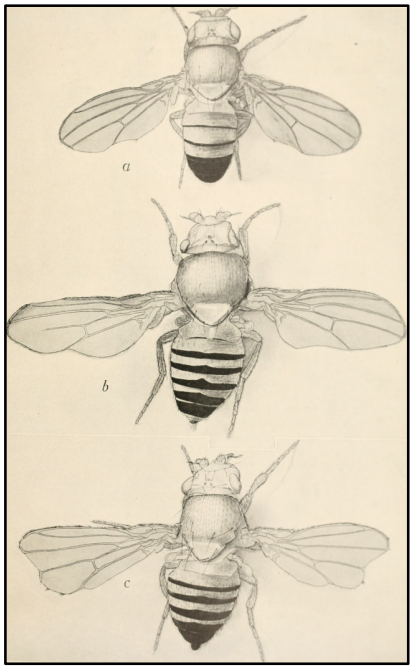
The effects of artificial light at night on the behaviour and physiology of *Drosophila melanogaster*

by

Lucy Katherine Ruth McLay



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Thesis Abstract

Artificial light at night is one of the most pervasive and least recognised anthropogenic pollutants. Its extent and intensity is expanding globally, at an estimated rate of 2.2% per annum, such that many urban and peri-urban animals no longer experience natural darkness. The majority of animal species have evolved under bright days and comparatively dark nights, with their physiology and behaviour synchronised to this rhythm. Accordingly, disruption to this cycle is linked to a suite of behavioural and physiological shifts across taxa. Experimental and correlational evidence documenting phenotypic responses to the presence of artificial light at night are accumulating but the underlying mechanisms driving the observed negative impacts associated with artificial light at night are not resolved. A likely reason is that light at night disrupts circadian rhythms and contributes to perturbed oxidative status through its interaction with the indolamine, melatonin, a key driver of circadian rhythm and powerful antioxidant. A major and currently untested gap in the literature is whether light at night can act as a driver of evolutionary change.

In this thesis, I investigated the short-term phenotypic impacts of ecologically relevant levels of artificial light at night on life history traits (**throughout Chapters 2 to 5**) and oxidative status (**Chapters 2 and 4**), using the model organism *Drosophila melanogaster*. I then used experimental evolution to explore whether artificial light at night can induce local adaptation in populations evolved over 15 to 25 generations (**Chapter 4**). Finally, using a melatonin dietary supplementation experiment, I attempted to investigate the relationship between artificial light at night and the melatonin pathway, thus hoping to demonstrate disruption to the melatonin pathway as a causal mechanism underpinning the observed phenotypic responses (**Chapter 5**).

My results demonstrated experimentally, that individuals exposed to artificial light at night have short-term phenotypic changes, with disrupted mating behaviour, reduced fecundity and size, altered development patterns and reduced longevity in *D. melanogaster* (**Chapters 2 to 5**). Additionally, I present novel (and somewhat counter intuitive) evidence, that artificial light at night is associated with lower levels of reactive oxygen species in ovaries (**Chapter 3**) and, aligned with this, reduced oxidative DNA damage in female flies (**Chapter 4**). In contrast, I found no evidence for such effects in males (**Chapters 3 and 4**). Despite potential for evolutionary change, I found little evidence for adaptation in most fitness traits after evolution

under artificial light at night (**Chapter 4**), with mating propensity the only life history trait to exhibit limited local adaptation. This suggests that the selective pressure of artificial light at night in the laboratory, may be weaker than anticipated. However, the fact that some level of adaptation was evidenced in relatively benign conditions, suggests that, in a more natural competitive environment, the selection pressure of light at night may be stronger.

I was unable to conclude whether melatonin is a potential mechanism driving phenotypic variation following exposure to light at night (**Chapter 5**), as the melatonin doses and design used, despite replicating a previous study, resulted in negative fitness consequences (reduced longevity under dark nights and disrupted eclosion regardless of light treatment), suggesting the melatonin supplementation was potentially toxic. Nonetheless, these data potentially support the importance of circadian disruption under artificial light at night as a driver of the observed negative effects. I propose that further research is warranted to test different melatonin concentrations and dosage duration at different life stages and at different times relative to circadian rhythm. This variation in dosage regimens may be able to better define its role (and the relevant contributions of circadian disruption and oxidative status) in the effects of artificial light at night.

This thesis demonstrates the negative impacts of artificial light at night in the model species, *D. melanogaster* and adds to the growing body of literature documenting taxa-wide damaging effects of artificial light at night on animal behaviour and physiology. As we continue to light our nights and reduce natural darkness globally, understanding the consequences and mechanisms behind the effects of artificial light at night is paramount to informed decision making around urban lighting strategies. Moreover, understanding the role of artificial light at night in driving evolutionary change, is of the utmost importance in maintaining wildlife stability in an increasingly urbanised world.

Declaration

This is to certify that:

(i) This thesis comprises only my original work towards my Doctor of Philosophy, except where otherwise stated in the Preface

(ii) Due acknowledgement has been make to all other material used

(iii) This thesis is fewer than 80,000 words excluding references, tables and figures

Signed

Lucy Katherine Ruth McLay

August 2019

Preface

I have structured this thesis into six chapters investigating the impacts of artificial light at night (ALAN) on the model invertebrate species *Drosophila melanogaster*. The thesis comprises of an introduction chapter, four data chapters and a general discussion. **Chapters 2 and 3** are peer-reviewed manuscripts that have been published and **Chapters 4 and 5**, are currently in preparation to be submitted for publication. Consequently, each data chapter is a standalone work that can be viewed independently from the rest of the thesis and therefore, there is some necessary repetition between chapters. I have attempted where possible to reduce the overlap.

I was principal contributor and primary author of all chapters. My supervisors, Therésa Jones and Mark Green, have been recognised for their invaluable contribution in all chapters and all other contributors are mentioned in the acknowledgements.

Funding

Funding for this research was provided by the Herman Slade Foundation, Grant number HSF 14/4 and internal University of Melbourne funding awarded to my supervisors.

Acknowledgements

First and foremost, I am incredibly grateful to my two supervisors. Therésa – thank you for your mentorship, insight into the heart of a problem and (annoyingly) good use of the English language. Mark – thank you for your unwavering support, nuanced ideas and good-humoured leadership. I have learnt so much from both you. It has truly been a journey and you have both guided me expertly.

I would like to thank the Jones lab group for their support, banter and insane conversations at lab coffee. Your humour has made such a difference to coping with much of the drudgery behind science. To my office-mates, thank you for listening to and commiserating with me when necessary and for providing support and comradery through my PhD years.

Others at The University of Melbourne I would like to thank are: Beth Finger for her technical input, reliable advice, calculation-backup and can-do attitude; my committee, Kath Hassell and particularly Charlie Robin, whose advice and friendly chats gave me perspective and confidence; Mark Elgar, you pointed me in the right direction at the beginning of my scientific endeavours and then again after my lengthy absence; and the Hoffmann lab, especially Jen Shirriffs, for my initial flies and husbandry advice for a complete novice.

At Monash, I would like to thank Damien Dowling and Venkatesh Nagarajan-Radha for ideas and practical input into ROS measurement.

I would like to thank my incredible wonderful husband, family and friends. Pat – your willingness to go down the PhD path, your constant support, encouragement, willingness to listen (or at least pretend to) all the time (and of course financial backing) are invaluable. Elena, Joseph and Philippa – thank you putting up with a mum who didn't do a PhD on a more charismatic animal and who knows way too much about the effects of artificial light at night on device-use before bed-time. Fiona – there is absolutely NO way I could have done this without you. Your care of the kids, willingness to do whatever was required and just general competence are highly appreciated. Mum and Dad – thank you for encouraging me (none-too gently in the case of Mum) to do a PhD and to my siblings who have particular insight into the PhD journey.

Finally, to the flies – sorry I killed so very many of you. I didn't enjoy doing it. It can be frustrating to work with small things that fly, but you mostly stayed where you were asked to.

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Chapter 1: Introduction

1.1 Artificial light at night as an environmental pollutant

Anthropogenic impacts on the environment are causing unprecedented declines in global biodiversity (Barnosky et al., 2011, Ceballos et al., 2015) through sustained habitat destruction, shifts in the distribution of invasive species, increased climate change and pollution (Novacek and Cleland, 2001). One of the most pervasive, yet potentially less well recognised, environmental pollutants is human-produced artificial light at night (ALAN), which is increasing rapidly in extent and intensity (Kyba et al., 2017), with increasing global urbanisation (Zhang, 2016). Moreover, scattered light in the form of sky-glow from cities and large brightly lit installations can be seen tens of kilometres away from the original light source (Kyba et al., 2011, Secondi et al., 2017). As a result, an increasing number of urban and peri-urban species never experience natural night-time darkness. Since animal physiology, behaviour and life history traits have evolved to synchronise to a predictable daily cycle of a bright day ($\leq$ 100,000 lx) and dark night ($\leq$ 2 lx) (Longcore and Rich, 2004, Gaston et al., 2014), one might expect ALAN to affect both the behaviour and physiology of organisms. Accordingly, over the past two decades, experimental and correlational studies have demonstrated the far reaching and almost exclusively negative consequences for plants and animals (including humans) of ALAN at the individual, population and ecosystem levels (for recent reviews see Gaston et al., 2013, Russart and Nelson, 2018).

1.2 The biological consequences of light at night

At the individual level, ALAN is associated with behavioural shifts in vertebrates, such as changes to daily activity patterns and foraging (Bird et al., 2004, Stone et al., 2009, Santos et al., 2010, Polak et al., 2011, Riley et al., 2012, Becker et al., 2013, Dominoni et al., 2013d, Pulgar et al., 2019), migratory behaviour (Witherington, 1992, Witherington and Martin, 1996, Poot et al., 2008), and reproduction and mating (Baker and Richardson, 2006, Titulaer et al., 2012, Dominoni et al., 2013c, Da Silva et al., 2015, de Jong et al., 2015, LeTallec et al., 2015, Robert et al., 2015). Until approximately five years ago, comparatively fewer studies had investigated ALAN induced behavioural effects in invertebrates, however a recent surge in

research has detailed evidence of effects of ALAN on movement and migration, with a 'flight-to-light' response reported in many species (reviewed in Desouhant et al., 2019), as well as changes to foraging patterns (both negative Luarte et al. (2016), van Langevelde et al. (2017), Farnworth et al. (2018) and positive Willmott et al. (2019)) and reproductive behaviour (van Geffen et al., 2015b, Firebaugh and Haynes, 2016, Botha et al., 2017, McLay et al., 2018, Owens and Lewis, 2018). At the broader scale, the impact of ALAN may extend beyond individuals. Studies suggest impacts on food webs (Sanders et al., 2015) and community composition (Davies et al., 2012, Davies et al., 2015, Hoelker et al., 2015, Grubisic et al., 2017, Manfrin et al., 2017), with the potential to threaten biodiversity (Hoelker et al., 2010, Knop et al., 2017). The effect of ALAN over multiple generations is currently a largely unexplored topic. It has been postulated that ALAN is a potential driver of evolution (Swaddle et al., 2015, Hopkins et al., 2018). However, to my knowledge, no evolution studies using ALAN as a sole selective agent (excluding other urban factors) have been undertaken.

ALAN is also associated with physiological changes. In humans, correlational evidence from epidemiological studies link ALAN with breast cancer (reviewed in Stevens, 2009), prostate cancer (Kloog et al., 2009) and colorectal cancer (Schernhammer et al., 2003). Experimental evidence from rodent models support this link (Blask et al., 2003, Anisimov et al., 2004, Blask et al., 2005, Cos et al., 2006) and also link exposure to ALAN with suppressed immunity (Bedrosian et al., 2011b), mood shifts (Bedrosian et al., 2011a) and obesity (Fonken et al., 2010, Aubrecht et al., 2015). Additionally, developmental studies suggest ALAN is likely to induce transgenerational effects (Cisse et al., 2017a, Cisse et al., 2017b). In invertebrates, recent studies have demonstrated a link between ALAN and shifts in development and size (Thakurdas et al., 2009, van Geffen et al., 2014, Durrant et al., 2018, Willmott et al., 2018), impaired immune function (Durrant et al., 2015), reductions in fecundity (McLay et al., 2017, Willmott et al., 2018), altered pheromone profile (van Geffen et al., 2015a), changes to oxidative status (McLay et al., 2018) and reduced longevity (McLay et al., 2017). The underlying mechanism causing the observed behavioural and physiological shifts associated with the presence of ALAN is unproven. However, experimental evidence, derived largely from vertebrate models, such as rats and mice, suggests that a link may be the negative effect of ALAN on the endogenous melatonin pathway (Nelson and Blom, 1994, Blask et al., 2002, Anisimov et al., 2004, Blask et al., 2005, Cos et al., 2006, Blask et al., 2009, Vinogradova and Anisimov, 2013, Schwimmer et al., 2014).

1.3 Melatonin as a potential mechanism promoting change

Melatonin (*N*-acteyl-5-methoxytryptamine) is an indolamine that is distributed almost ubiquitously in nature and is present in all major taxa of organisms (Vivien-Roels and Pevet, 1993, Hardeland and Poeggeler, 2003, Reiter et al., 2013). Its production is inhibited by light (Reiter et al., 2010) resulting in a rhythmic daily cycle that peaks during periods of darkness in most animals (Tan et al., 2010). When light falls on specialised photopigments in the eye, it entrains the master clock located in the suprachiasmatic nucleus (mammals) (Navara and Nelson, 2007) or in the brain (insects) (Dubowy and Sehgal, 2017). The clock in turn, stimulates the production of melatonin during darkness when electrical activity is low (Pandi-Perumal et al., 2006). Melatonin from the brain circulates to other peripheral tissues and modulates a diverse range of physiological actions (for reviews, see Pandi-Perumal et al., 2006, Tan et al., 2010), although the master clock can also influence tissues independently of melatonin, by direct neuronal control (Buijs et al., 2003). In vertebrates, the main site of melatonin secretion is the pineal gland, and similarly in invertebrates it is found in highest concentrations in the brain, eye and optic lobes (Vivienroels et al., 1984, Finocchiaro et al., 1988, Abran et al., 1994, Itoh et al., 1995a, Itoh et al., 1995b, Bembenek et al., 2005, Yang et al., 2007). However, it is also present and synthesised in other cells and organs (Tosini and Menaker, 1998, Pandi-Perumal et al., 2006, Tan et al., 2010, Mukherjee et al., 2014, Reiter et al., 2013).

As production of melatonin is controlled by light, melatonin acts as a chronobiotic, signalling day length and season change and controlling behaviour and physiological processes associated with circadian rhythms (Zawilska, 1996, Arendt and Skene, 2005, Reiter et al., 2010, Hardeland, 2013). However, a less well recognised function of melatonin is as a powerful free radical scavenger (for reviews see Reiter et al., 2000, Tan et al., 2002, Hardeland, 2005, Anisimov et al., 2006, Peyrot and Ducrocq, 2008). As part of normal cell metabolism, oxygen undergoes oxidative phosphorylation generating various oxygen by-products that are potentially highly reactive (Dowling and Simmons, 2009). These by-products are known as free radicals or reactive species, one group of which, is reactive oxygen species (ROS) (Dowling and Simmons, 2009). A certain level of ROS is required for normal cell function (Droge, 2002), however when ROS gets out of balance with an organism's antioxidant capacity, it leads to oxidative stress and ROS can interact with cellular macromolecules, compromising cellular function and potentially lead to DNA damage and/or cell death (Reiter et al., 2000). Thus,

melatonin is a vital hormone not only in controlling daily and seasonal rhythms, but also in regulating cell maintenance and health.

1.4 Thesis objectives

Currently, there is no direct evidence for the causal link between the effects of ecologically relevant ALAN intensity, melatonin and oxidative damage, although several studies have demonstrated a correlation between dim ALAN (defined here as $\leq$ 10 lx, which is typical of urban streets) and reduced levels of circulating melatonin (Brainard et al., 1982, Dominoni et al., 2013a, Bruning et al., 2018, Dimovski and Robert, 2018). Additionally, the focus of the effects of ALAN has been on individual animals within a single generation, yet understanding the effects of ALAN over multiple generations and that ALAN may drive evolutionary change in populations, is vital to the management of wildlife stability in the future. Redressing these shortfalls requires longitudinal studies over multiple generations.

Accordingly, my thesis has four main objectives (as presented in Figure 1-1):

1. To explore whether ALAN at different ecologically-relevant intensities has an effect on life history traits.
2. To determine if there is correlation between dim ALAN and oxidative status.
3. To investigate whether dim ALAN can induce local adaptation.
4. To investigate melatonin as a potential mechanism behind the effects of dim ALAN on life history and physiological traits.

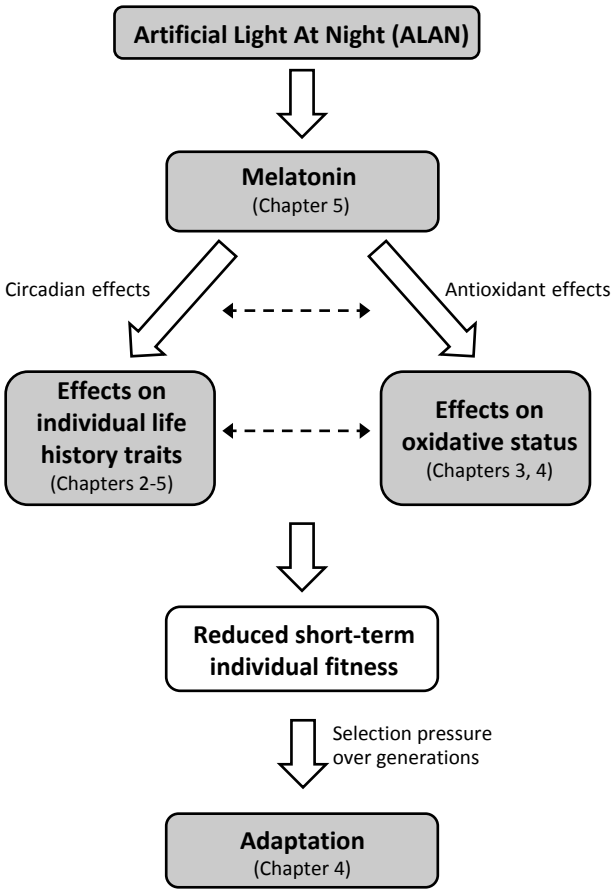

Figure 1-1. Schematic diagram of potential effects, mechanisms behind and possible interactions between, the effects of artificial light at night (ALAN) in *Drosophila melanogaster*. White block arrows depict potential mechanisms, dotted lines show potential interactions, grey boxes represent thesis objectives and chapters. ALAN may reduce endogenous melatonin synthesis, which potentially results in circadian shifts and reductions in antioxidant capacity. Together, or separately, these effects negatively affect life history traits and lead to reduced individual fitness and may consequently lead to local adaptation over multiple generations.

1.5 Study species

In this thesis, I experimentally manipulated the presence of light at night in laboratory experiments and assessed phenotypic and evolutionary responses in a well-defined model invertebrate species. My study species of choice, *Drosophila melanogaster*, is ideal as it is

arguably one of the best studied and understood animal species in medicine and ecology; has a rapid generation time (approximately 10 days from egg to adult at 28^{o}C); can be cultivated easily under laboratory conditions; has well characterised circadian rhythms; and, melatonin has been identified (Finocchiaro et al., 1988) and is linked with improved survival when administered as a dietary supplement (Coto-Montes and Hardeland, 1999, Bonilla et al., 2002, Bonilla et al., 2006, Teran et al., 2012, Medina-Leendertz et al., 2014). The population used in my thesis originated from wild caught individuals from the Yarra Valley in Victoria, Australia (map reference: -37.686908, 145.457438) that had been maintained in the laboratory under standard conditions for 27 generations.

1.6 Thesis outline

To address my first objective: whether ALAN induced shifts in life history traits in *D. melanogaster* and whether there was a dose response to variation in nocturnal light intensity, in **Chapter 2,** I reared three generations of flies under varying ecologically relevant, light at night treatments (0 lx = dark night, 1 lx = equivalent to approximately three times full moon, 10 lx = approximately equivalent to a street light, 100 lx = approximately equivalent to a brightly lit urban space) and then explored subsequent variation in three key fitness traits (growth, reproduction and survival). My results demonstrated effects across the fitness measures of reproduction and survival across all ALAN levels (from 1 through to 100 lx), but 10 lx and 100 lx induced the most change and were comparable. Thus, there was no dose dependant response and given this, thereafter, all of my experiments compared 0 lx to 10 lx ALAN (**Chapters 3 to 5**) as these two measures were representative of rural and urban nocturnal light levels respectively (Gaston et al., 2014). In **Chapter 3**, I explored the effects of lifetime exposure to ALAN on different life history traits (mating behaviour and lifetime oviposition patterns) and on the physiological parameter of ROS levels (my second objective) in heads or ovaries of 6-, 23- and 26-day-old females that had been reared under ALAN treatment for three generations.

The effects of ALAN on life history traits demonstrated in **Chapters 2 and 3**, suggest that ALAN may substantially reduce the fitness of individual flies and could therefore act as a selective pressure promoting local adaptation. To explore this and address my third research objective, in **Chapter 4,** I used an experimental evolutionary approach over 25 generations, using five

replicate populations of flies evolved under either 0 lx or 10 lx ALAN. I used a reciprocal transplant approach to compare the life history traits of mating behaviour, oviposition, longevity (generation 17) and to explore variation in size and oxidative DNA damage (generation 27).

In **Chapter 5**, I addressed my fourth research objective of whether melatonin may be a potential mechanism underpinning the observed phenotypic shifts observed under dim ALAN. Here, I reared *D. melanogaster* for three generations under either 0 lx or 10 lx ALAN treatments and simultaneously provided lifetime dietary supplementation with melatonin at three previously published concentrations used for *D. melanogaster* (0 μg/ml, 50 μg/ml or 100 μg/ml). I then assayed the life history traits of female fecundity, offspring development, size and adult longevity to assess whether melatonin can modulate the effects of dim ALAN.

Finally, in **Chapter 6**, I synthesise the results of my experimental chapters (**Chapters 2 to 5**), placing my findings into context, outlining their implications and suggesting avenues for future research. Significantly, the experimental designs in my thesis have meant that I was able to repeatedly measure some of the same fitness and physiological traits in different experiments, producing result replication, a lack of which, is an important and currently highly discussed shortcoming of the modern science publication process (Baker, 2016). The result of this replication, is that I can be confident that ALAN's numerous phenotypic effects in *D. melanogaster* are robust and not an artefact of a single experiment.

Overall this thesis aims to determine the range and level of effects of ALAN on behaviour and physiology in the model species, *D. melanogaster*. In doing so, I hope to elucidate the broader implications of ALAN as an environmental pollutant with the potential to affect urban wildlife. Finally, I propose to look to the future, to determine if there is the potential for adaptation to ALAN. Given that ALAN is expanding rapidly world-wide, I hope that my research provides avenues for future research into the mechanisms behind the effects of ALAN and therefore potential measures to mitigate its impact on global biodiversity.

Original paper

Chapter 2:

Chronic exposure to dim artificial light at night decreases fecundity and adult survival in *Drosophila melanogaster*

by

Lucy Katherine McLay, Mark Philip Green and Therésa Melanie Jones

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2.1 Abstract

Artificial light at night (ALAN) is expanding in geographical range and increasing in intensity to such an extent that species living in urban environments may never experience natural darkness. The negative ecological consequences of artificial night lighting have been identified in several key life history traits across multiple taxa (albeit with a strong vertebrate focus); comparable data for invertebrates is lacking. In this study, we explored the effect of chronic exposure to different night-time lighting intensities on growth, reproduction and survival in *Drosophila melanogaster*. We reared three generations of flies under identical daytime light conditions (2600 lx) and one of four ecologically relevant ALAN treatments (0, 1, 10 or 100 lx), then explored variation in oviposition, number of eggs produced, juvenile growth and survival and adult survival. We found that, in the presence of light at night (1, 10 and 100 lx treatments), the probability of a female commencing oviposition and the number of eggs laid was significantly reduced. This did not translate into differences at the juvenile phase: juvenile development times and the probability of eclosing as an adult were comparable across all treatments. However, we demonstrate for the first time a direct link between chronic exposure to light at night (greater than 1 lx) and adult survival. Our data highlight that ALAN has the capacity to cause dramatic shifts in multiple life history traits at both the individual and population level. Such shifts are likely to be species-specific, however a more in depth understanding of the broad-scale impact of ALAN and the relevant mechanisms driving biological change is urgently required as we move into an increasing brightly lit future.

2.2 Introduction

The majority of animal species have an evolutionary history that involves a predictable daily cycle, characterised by a bright day (up to 200,000 lx) and a dark night (between 0.00003 and 0.03 lx) (Longcore and Rich, 2004, Gaston et al., 2014). Accordingly, species have evolved physiological and behavioural traits that are synchronised to this light-dark daily rhythm and corresponding seasonal changes (Navara and Nelson, 2007). However, recent anthropogenic alteration to the photic urban environment, in the form of artificial lighting from streetlights and other illuminated structures, has caused an unprecedented reduction in the true dark period within this daily cycle (Cinzano et al., 2001, Falchi et al., 2016). Both the degree and intensity of artificial light at night (ALAN) in urban regions have increased significantly over the past century (Cinzano et al., 2001), resulting in nights that are substantially brighter than at any other time point in evolutionary history. Organisms living within ALAN affected environments thus rarely (if ever) experience natural levels of night-time illumination (Bennie et al., 2015).

Disruption to the nocturnal photic environment is linked with corresponding biological shifts at the individual, population and possibly ecosystem levels (for reviews see Tierney et al., 2017b, Jones et al., 2015, Gaston et al., 2014, Navara and Nelson, 2007). Increasingly, evidence suggests that a remarkably low threshold of night light intensity can generate detectable biological change. Recent field studies in birds and mammals have revealed that chronic exposure to less than 10 lx correlates with reductions in individual fitness traits, such as reproduction and juvenile growth (Dominoni et al., 2013b, Dominoni et al., 2013e, LeTallec et al., 2015, Robert et al., 2015, Raap et al., 2016). Furthermore, epidemiological studies in humans demonstrate a correlation between dim ALAN exposure and cancer risk (for a review see Cho et al., 2015). Similarly, laboratory studies on rodents report a positive relationship between the presence of dim ALAN and tumour growth (Cos et al., 2006, Cho et al., 2015), immune suppression (Bedrosian et al., 2011b) and obesity (Fonken et al., 2010, Aubrecht et al., 2015).

Comparatively few studies have investigated whether exposure to dim night lighting results in comparable reductions in fitness in invertebrates. Typically, invertebrate studies have compared a normal day-night: light-dark environment with the presence of constant light. Nonetheless, they reveal a negative effect of the presence of constant light on fecundity and

longevity in *D. melanogaster* (Kouser et al., 2014), an effect on development in *D. melanogaster* (Sheeba et al., 1999), a reduced capacity to up-regulate the immune response in the cricket *Teleogryllus commodus* (Durrant et al., 2015), and a reduction in clutch size in the ant *Camponotus compresses* (Lone and Sharma, 2008). Moreover, the few studies that have exposed invertebrates to bright day with dim ALAN conditions indicate that the biological effects of low ALAN intensities may be taxa-wide and far reaching in terms of their fitness consequences. For example, field studies of moths (*Operophtera brumata*) and fireflies (*Photuris versicolour*) demonstrate that females in dimly lit ALAN environments have a reduced likelihood of successfully mating (van Geffen et al., 2015b, Firebaugh and Haynes, 2016). Similarly, laboratory experiments have revealed that, in the presence of dim night-lighting, sexually producing female aphids (*Megoura viciae*) switch to an asexual mode of reproduction (Sanders et al., 2015); moths (*Mamestra brassicae*) have accelerated juvenile development (van Geffen et al., 2014); the beach amphipod (*Orchestoidea tuberculata*) has decreased growth (Luarte et al., 2016); and, the tropical fruit fly *Drosophila jambulina* has an unusually arrhythmic timing of eclosion (Thakurdas et al., 2009). A characteristic of the majority of studies (whether vertebrate or invertebrate) is they typically explore a single fitness trait and have thus investigated fitness effects within rather than across generations.

Here, we investigate whether the presence of ALAN at the ecologically relevant levels of 0 lx (equivalent to darkness), 1 lx (three times full moonlight), 10 lx (average urban street lighting) or 100 lx (bright urban night lighting) affects the three key life history traits of reproduction, development and longevity in a model invertebrate, *D. melanogaster*. The four ALAN intensities were chosen because they occur in urban environments and have been demonstrated to disrupt biological processes (Bruning et al., 2015). *Drosophila melanogaster* is an ideal model organism to investigate the effects of ALAN as it has a rapid life cycle, thus allowing exploration of multi-generational effects. It also inhabits both areas of natural night-time darkness and ALAN affected environments (Keller, 2007), but whether individuals and populations respond differently in these different ALAN environments is unknown. Moreover, many behaviours in *D. melanogaster* are governed by circadian rhythm, including eclosion, courtship, mating and oviposition (Peschel and Helfrich-Forster, 2011). In line with previous studies in invertebrates, we predict that the presence of ALAN during development will have a negative effect on fecundity, growth and survival. Furthermore, assuming a dose-dependent effect of the presence of light at night, we expect that higher levels of ALAN intensity will have the greatest impact.

2.3 Materials and Methods

2.3.1 Fly stocks

A stock population of *D. melanogaster* was derived from 100 adult females and 50 adult males collected in late April 2015 from Oak Ridge Winery in the Yarra Valley, Victoria, Australia (-37.686908, 145.457438). Field-caught flies were transferred to glass stock bottles (135mm x 55mm) and maintained on a standard *Drosophila* diet of Bloomington's cornmeal medium (Brent and Oster, 1974) in a climate controlled laboratory at 25°C, 50% humidity and 12:12 hour light:dark cycle. Adults were subsequently maintained in stock bottles at a density of approximately 50 male and 50 female flies for two to three days before being removed. Emerging adult offspring from each bottle were transferred to a fresh stock bottle of medium and allowed to mate and oviposit for two to three days. After 20 generations, five stock bottles were transferred to an incubator (retrofitted Westinghouse: model number WRM4300WB-R) at 28 ± 1°C, approximately 50% humidity, with a 12:12 hour light (2600 lx, 6800 kelvin) / dark (0 lx) cycle) and flies were maintained in the same manner as above for a further seven generations to acclimatise them to laboratory incubator conditions of 12:12 hour day/night prior to the commencement of the experiment.

2.3.2 Experimental flies

After seven generations, we collected 20 newly emerged (virgin) female and male flies from each stock bottle under light CO_2 anaesthesia. To ensure equal contribution of stock populations to experimental vials, one female and one male from each stock bottle were placed in each of 20 *mating vials* (102mm high x 25mm diameter) containing Bloomington's cornmeal medium (n = 5 males and 5 females per vial). The vials were then divided equally between four ALAN treatments, such that stock flies were distributed evenly throughout vials and ALAN treatments.

2.3.3 Light treatments

We created the four light treatments using Westinghouse incubators (as above) retro-fitted with LED lights (day-time lights – SUPER-FLEX cool white, manufactured by World of Thought; night-time lights – DÉCOR-FLEX 5 cool white, manufactured by World of Thought). Each ALAN treatment had an identical daytime lux (2600 lx - equivalent to an overcast day, 6800 kelvin)

but varied in their night time lux (0, 1, 10 or 100 lx, 5900 kelvin). Light treatments were configured to 12 hour day: 12 hour night daily photic cycle. To ensure no incubator bias, flies and their corresponding ALAN environment were rotated between incubators every two to three days.

2.3.4 Effect of ALAN on reproductive fitness

We used six-day old *Drosophila* females to assess variation in oviposition (*sensu* Gruwez et al., 1971, McCabe and Birley, 1998). To confirm the egg-laying pattern of our female population was consistent under the different light treatments and thus our sampling of six-day old females was not biasing the data collected, we conducted a preliminary assay of oviposition for day two, four and six females. These data (given in Results) confirm the appropriateness of our use of six-day old females for the subsequent oviposition experiment.

Following our preliminary trials, we maintained groups of five female and five male flies (see *Experimental flies* above) in *mating vials* for five days, with a single change of medium at day two to three. At day six, males were discarded and surviving females were each transferred to an individual *oviposition vial* (102mm high x 25mm diameter), containing a plastic spoon (volume 3ml) filled with Bloomington's cornmeal medium that that had been dyed blue with food dye (Queen^{TM}) to increase egg visibility. Females were left for 23 hours to oviposit after which time they were discarded and the number of eggs laid by each female was counted.

2.3.5 Effect of ALAN on offspring development (success and rate)

Within each of the four light treatments, the five females that laid the most eggs were identified; data from all other females and their egg batches were recorded but flies and eggs were subsequently discarded from the study. We selected 20 eggs at random from each of the five identified egg batches and transferred them in batches of four to one of five *offspring vials* (dimensions as above). Thus, each of the five females' egg batches contributed equally to all *offspring vials* (resulting in 20 eggs per *offspring vial*). *Offspring vials* were returned to their original light treatment and left until all offspring had emerged as adults, or for a maximum of seven days after the first fly had eclosed from a given vial. *Offspring vials* were checked daily within an hour of the commencement of incubator day and the number of pupae and adults noted. The time taken between the oviposition date and adult eclosion was defined as juvenile development time.

2.3.6 Effects on the F_2 generation

To explore effects of light treatment on oviposition and development over a further generation, an F_2 population was established from the adult flies that emerged from the F_1 *offspring vials*. As for the parental flies, five virgin females and five virgin males were removed from each *F_1 offspring vial* and placed together in an *F_2 mating vial*. Flies were allowed to mate, females to oviposit, eggs batches counted, eggs transferred to *F_2 offspring vials* and *F_2 offspring vials* monitored in an identical manner to their parents.

2.3.7 Effect of ALAN on survival and size

To explore the effect of light treatment on adult survival in the F_1 generation, a virgin female and a virgin male was removed from each of the *F_1 offspring vials* (n= 25 vials) and a mated female and a mated male was removed from each of the *F_1 mating vials* following five days of mating opportunity (see above - *Effects on the F_2 generation*) (n = 25). Flies were transferred individually to vials containing standard medium and then checked every one to two days until death. Adult flies were transferred to a new vial weekly. Upon death, fly size was estimated by removing the left wing and measuring wing length from the intersection of the anterior cross vein and the longitudinal vein 3 to the intersection of longitudinal vein 3 with the distal wing margin as per Partridge et al. (1987). Wings were photographed under a compound microscope (Olympus BX50) with attached camera (Olympus U-TV0.63XC) and measurements made using Image J software (NIH).

2.3.8 Statistical analysis

The experiment was conducted in three blocks, resulting in 25 replicates overall. Analyses were performed using JMP version 12.1.0 (SAS institute, NC, USA). Prior to analysis, data were assessed for normality. Unless otherwise stated, we used generalized linear mixed models (GLMM) with a normal error distribution and vial and block included as random effects in all models; where data could not be appropriately transformed, we used non-parametric Kruskal Wallis tests (for models of juvenile development time and the proportion of pupae eclosed). Finally, we used the proportional hazard survival platform to assess variation in adult survival. We included light treatment as a factor in all models and, where appropriate, generation, sex, juvenile development time, size and number of adults eclosed from the offspring vial, as well as interactions between these as variables. The significance of parameters was assessed using

hierarchical backwards stepwise deletion, dropping terms from the model (except the main parameter of interest, light treatment) where $P > 0.10$. Unless otherwise stated all data presented are means ± standard errors and the level of statistical significance was taken as $P < 0.05$.

2.4 Results

2.4.9 Confirmation of the appropriateness of six-day old females

Our preliminary experiment confirmed the appropriateness of using six-day old females to test the effect of ALAN on oviposition. We found an effect of light treatment on the number of eggs laid (mean ± standard error of eggs laid per female per vial for 0 lx females = 12.11 ± 1.09; 1 lx = 7.85 ± 0.97; 10 lx = 5.10 ± 1.02; 100 lx = 8.04 ± 0.95; $F_{3,48} = 8.17$, $P < 0.001$): females from all other light treatments laid less eggs than 0 lx females (post hoc Tukey's tests: comparing 0 lx females with each of the other treatments: all $P < 0.05$; all other comparisons, $P > 0.05$). There was no main effect of female age on the number of eggs laid ($F_{3,48} = 0.001$, $P = 0.97$) and no significant interaction between female age and light treatment ($F_{3,48} = 2.7$, $P = 0.06$).

2.4.10 Effect of ALAN on oviposition

In the main experiment, the probability of a female commencing oviposition (laying at least one egg) varied with light treatment ($P < 0.001$; Table 2-1a): females exposed to the treatments with light at night (1, 10 and 100 lx) were less likely to commence oviposition than females in the 0 lx control treatment (post hoc comparisons between 0 lx females and each of the other light treatments: $P < 0.05$; all other comparisons $P > 0.05$). The number of eggs laid by ovipositing females (those females that laid at least one egg) also varied between light treatments ($P < 0.001$; Table 2-1b). Similarly, females from all of the light at night treatments laid fewer eggs than the 0 lx control (Table 2-1b; Figure 2-1). There was no difference in either the probability of oviposition or the number of eggs laid between generations F_0 and F_1 (both $P > 0.10$) and thus generation was dropped from each of these models.

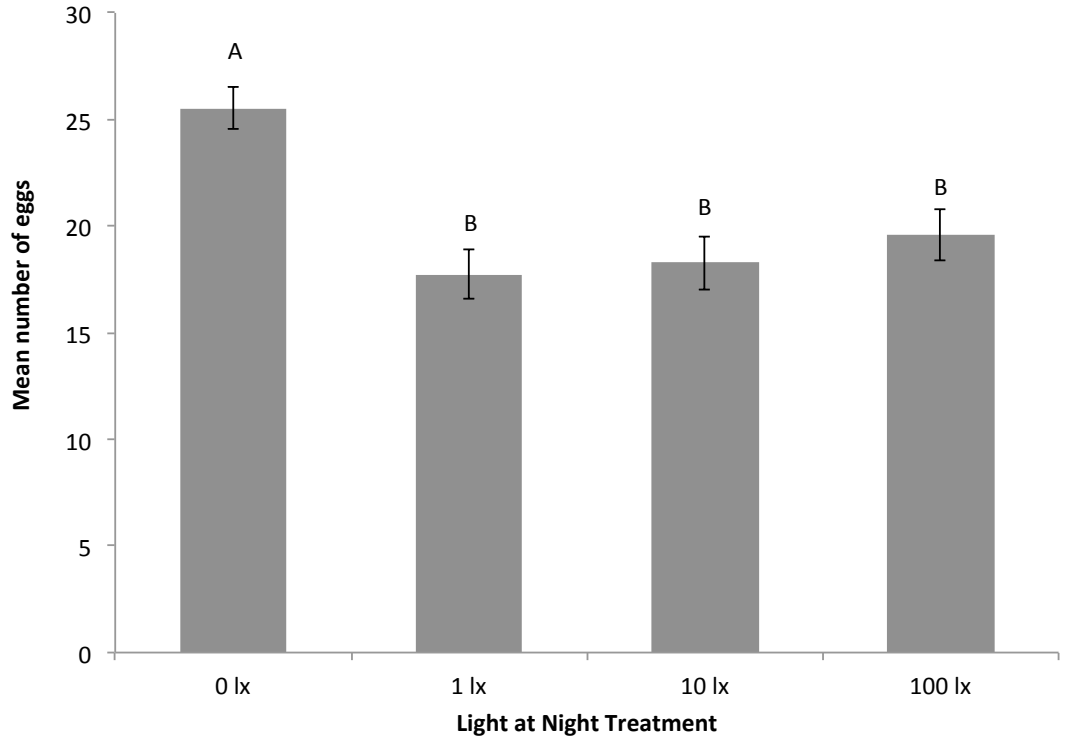

Figure 2-1 The mean number of eggs laid by ovipositing females under each of the four night time light intensities. Errors bars represent standard errors about the mean. Different superscript letters denote significant differences between groups ($P < 0.05$). n = 145 (0 lx); 147 (1 lx); 155 (10 lx); 154 (100 lx).

2.4.11 Effect of ALAN on offspring development

We found little evidence that chronic exposure to light at night affected the juvenile phase of the life cycle. There was also no variation across light treatments in the number of eggs reaching pupation ($P = 0.10$), the proportion of pupae that eclosed as adults ($P = 0.24$) or the number of adults eclosed ($P = 0.64$) (Table 2-1c, d, and e respectively). Similarly, juvenile development time (taken as the number of days from oviposition to eclosion) was comparable across the light treatments ($P = 0.56$, Table 2-1f) but was longer in the second compared to the first experimental generation ($P = 0.005$; Table 2-1f).

2.4.12 Effect of ALAN on adult survival and size

While exposure to dim light at night had limited effect on the juvenile phase of the cycle, adult survival varied significantly ($P = 0.003$; Table 2-1g). Post hoc models revealed that flies in the

10 and 100 lx treatments, died quicker than 0 lx flies, but 1 lx flies survived equally as long as the 0 lx flies. (Table 2-1g,

Figure **2-2**). Male flies survived longer than female flies (P = 0.04). Flies in the mated and unmated treatments survived equally well (P = 0.77) and thus mating status was dropped from the final model. Wing length was comparable across light treatments (P = 0.10, Table 2-1h) but females had longer wings than males (P < 0.001).

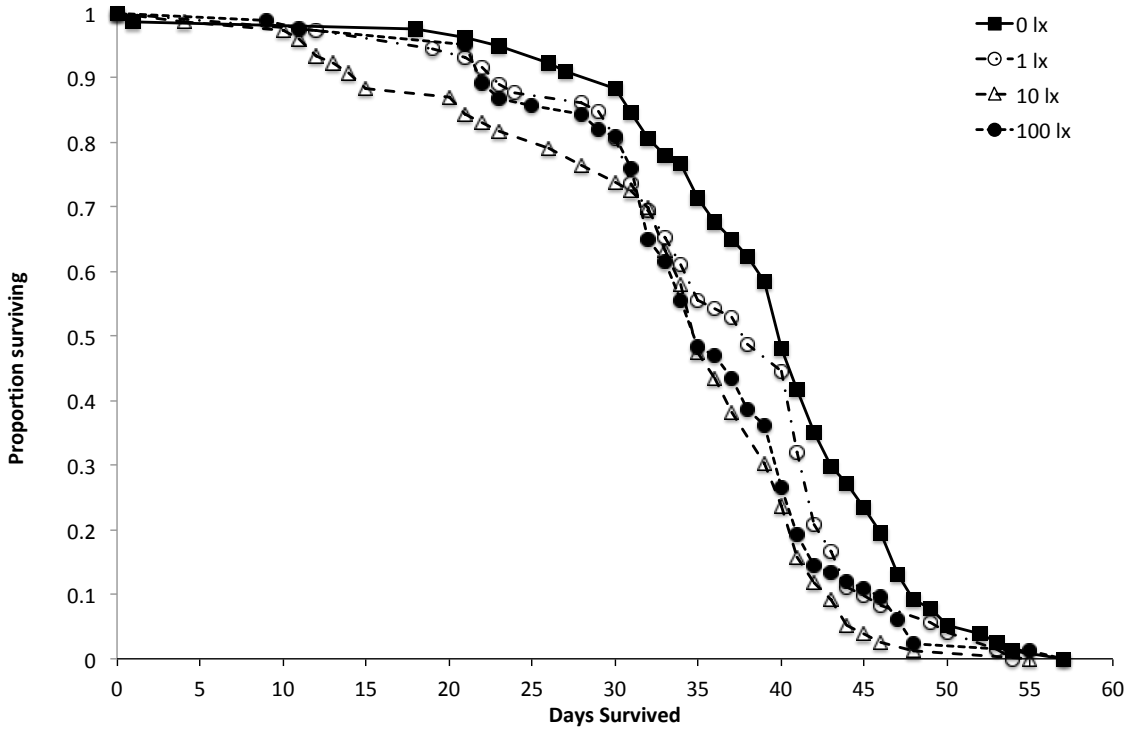

Figure 2-2 Survival curve calculated from predicted means for individuals kept under different night time light treatments. P = 0.003: 0 lx significantly different to 10 and 100 lx, but not significantly different to 1 lx. n = 32 (0 lx); 29 (1 lx); 32 (10 lx); 30 (100 lx).

Table 2-1. Statistical models exploring the effect of artificial light at night (ALAN) on life history traits in *D. melanogaster.* a) Number of females commencing oviposition; b) Number of eggs per female; c) Number of eggs reaching pupation; d) Proportion of pupae reaching eclosion; e) Number of adults eclosed; f) Juvenile development time; g) Number of adult days survived; h) Wing length (mm). Below each life history trait are only those variables found to be significant ($P < 0.05$) from the analysis. All statistics are mean ± standard error except were indicated (* number reported. T median; interquartile range reported). Different subscript letters denote significant differences between treatment groups.

Model Parameters	**Mean ± Standard Error ALAN treatment**				**Statistic, P value**
	0 lx	**1 lx**	**10 lx**	**100 lx**	
*a) Number of females commencing oviposition**					
Light treatment	$130/145_A$	$109/147_B$	$112/155_B$	$106/154_B$	**$\chi^2 = 20.62, P < 0.001$**
b) Number of eggs per female					
Light treatment	$25.5 \pm 0.98_A$	$17.7 \pm 1.17_B$	$18.2 \pm 1.27_B$	$19.5 \pm 1.19_B$	**$F_{3,457} = 10.76, P < 0.001$**
c) Number of eggs reaching pupation					
Light treatment	15.5 ± 0.39	15.8 ± 0.29	15.2 ± 0.60	16.6 ± 0.38	$F_{3,199} = 2.14, P = 0.10$
d) Proportion of pupae eclosed[T]					
Light treatment	0.95; 0.92 - 1.00	1.00; 0.93 - 1.00	1.00; 0.95 - 1.00	1.00; 0.89 - 1.00	$\chi^2 = 4.21, P = 0.24$
e) Number of adults eclosed					
Light treatment	15.0 ± 0.43	15.4 ± 0.35	15.5 ± 0.52	15.8 ± 0.43	$F_{3,199} = 0.56, P = 0.64$
f) Juvenile development time (days)[T]					
Light treatment	9.00; 9.00 - 9.00	9.00; 9.00 - 9.00	9.00; 8.50 - 9.00	9.00; 9.00 - 9.00	$\chi^2 = 2.08, P = 0.56$
Generation					**$\chi^2 = 8.03, P = 0.005$**
g) Adult survival (days)[T]					
Light treatment	$40; 35 - 45_A$	$38; 31 - 42_{AB}$	$35; 30 - 40_B$	$35; 32 - 41_B$	**$\chi^2 = 14.16, P = 0.003$**
Sex					**$\chi^2 = 4.42, P = 0.04$**
h) Wing length (mm)					
Light treatment	1.35 ± 0.01	1.37 ± 0.01	1.36 ± 0.01	1.35 ± 0.01	$F_{3,300} = 2.14, P = 0.10$
Sex					**$F_{1,300} = 868.3, P < 0.001$**

2.5 Discussion

Our study resulted in three key findings. First, chronic exposure to dim night lighting (1, 10 and 100 lx) correlated with a reduction in the probability of a female commencing oviposition and, for those females that did oviposit, a reduction in the number of eggs laid. Second, contrary to other studies in invertebrates (van Geffen et al., 2014), there was no effect of the presence of dim light at night on the duration of the juvenile phase of the life cycle. Finally, and perhaps most importantly, we found that the presence of dim night lighting significantly reduced adult survival. Combined our data demonstrate a substantial negative impact of the presence of dim night lighting on key life history traits in *Drosophila melanogaster* that may have individual and population level consequences.

On average, the presence of dim night lighting resulted in a 20% reduction in the number of females commencing oviposition (90% of 0 lx females commenced oviposition compared to approximately 70% for the ALAN treatment groups; Table 2-1a) and a similar percentage reduction in the number of eggs laid by females that did oviposit (0 lx females laid on average 25 eggs compared to an average of 20 eggs for females from the ALAN treatment groups; Table 2-1b). Our preliminary experiments confirm that the observed variation in oviposition data was unlikely due to differences in oviposition strategies (i.e. 0 lx females did not commence laying later or lay fewer eggs earlier compared to females from the other ALAN treatments; rather the number of eggs laid by 0 lx females was consistently higher). Thus, these data likely represent real fitness costs for female *D. melanogaster* living in the presence of dim night lighting and, assuming these patterns persist as a female ages, this may severely reduce lifetime reproductive success.

The variation in oviposition with light treatment may arise because dim ALAN represents a sub-optimal mating environment, as was observed in moths and fireflies (van Geffen et al., 2015b, Firebaugh and Haynes, 2016). In *D. melanogaster*, mating stimulates egg production and subsequently increases oviposition rate (Wolfner, 2002, Gillott, 2003). It is therefore conceivable that the observed decrease in both the percentage of individuals commencing oviposition and the number of eggs laid in the ALAN treatments may have arisen due to a lower female mating frequency in these treatment groups. We did not observe flies mating directly but suggest that this is unlikely for two reasons. First, the peak time for mating in *D. melanogaster* is during daylight hours (3-6 hours after dawn, Sakai and Ishida, 2001), therefore

the presence of night lighting is unlikely to have a direct impact on the key time period during which mating occurs. Second, mating success is negatively correlated with survival in *D. melanogaster* (Fowler and Partridge, 1989, Chapman et al., 1995) and thus, if the lower fecundity observed for ALAN treatment group females were a result of decreased mating success, we would expect to see a concomitant increase in survival in these treatment groups; this was not the case. On the contrary, adult survival was significantly higher for females (and males) reared in the absence of night lighting despite a higher female reproductive output.

An alternative explanation for the variation in oviposition data is that ALAN directly affects oviposition behaviour *per se*. *Drosophila melanogaster* has a dusk or early night maximum in terms of the number of eggs laid (Howlader and Sharma, 2006) and thus the presence of constant dim night lighting may change the duration of time optimal for oviposition. However, if true, then we would perhaps expect 0 lx females to lay fewer eggs as they were exposed to less dim light during their 24 hour cycle, which again is in direct contrast to our findings. Furthermore, *D. melanogaster* have been shown to retain oviposition rhythmicity even under constant bright light conditions (Sheeba et al., 2001) and thus the mechanism by which low intensity night lighting would create the observed shifts in egg laying remains uncertain.

Chronic exposure to dim night lighting had limited effect on the juvenile phase of the lifecycle but resulted in a substantial reduction in adult longevity: males and females exposed to dim night lighting (10 lx and 100 lx) survived for five days less than adults in the 0 lx control. In practice, this represents a 12% reduction in adult life span and is the first study to find a direct effect of chronic exposure to dim light at night on longevity in adults. These results are corroborated by the studies to date under constant bright light conditions, which have demonstrated a decrease in adult longevity (Sheeba et al., 2000, Kouser et al., 2014) but no difference in pre-adult survival (Sheeba et al., 2000). The reduction in overall survival coupled with reductions in female fecundity mean that the presence of night lighting may result in a substantial fitness cost for individuals and populations living in urban environments.

The fact that our study was conducted in a relatively benign laboratory environment means the biological impact may be substantially greater in a more malign environment. However, we note that even our dim light treatments were sufficiently stressful to generate biological difference across individuals and that combined, these potentially result in a substantial reduction in fitness at the population level. We further highlight the fact that, as a species, *D.*

melanogaster appears to respond rapidly to change: the time for development increased between the first and second experimental generations (most likely as a result of selecting females with the most eggs in each generation).

Notwithstanding potential shifts in behaviour, there are a number of physiological mechanisms that are likely disrupted in the presence of artificial light at night and which could have generated the observed differences in fecundity and survival across light treatments. One possibility is that ALAN affects the endogenous production of melatonin, a known driver of circadian rhythm and powerful antioxidant (for reviews see Arendt and Skene, 2005, Reiter et al., 2010). Melatonin is ubiquitously present across a range of taxa, including vertebrates (Tan et al., 2010) and invertebrates (Vivien-Roels and Pevet, 1993, Jones et al., 2015), including *D. melanogaster* (Finocchiaro et al., 1988). The production of melatonin is photosensitive and has a rhythmic daily cycle, typically peaking at night in most taxa (Arendt and Skene, 2005). Significantly, melatonin production is also sensitive to artificial light and thus in the presence of even dim artificial night lighting (from 0.03 – 1.1 lx), circulating melatonin levels are dramatically reduced (Brainard et al., 1982, Figueiro et al., 2011, Dominoni et al., 2013a) causing potential disruption to both the circadian cycle and oxidative stress (Pandi-Perumal et al., 2006).

The observed differences in reproductive output highlighted in our experiment could be attributed to disruption of the circadian rhythm by light-imposed disruption to the melatonin pathway (for mechanism by which this could occur see Zawilska et al., 2009). Similarly, ALAN induced reductions in melatonin levels may contribute to higher levels of oxidative stress that in turn may affect gamete production and physiology (Metcalfe and Alonso-Alvarez, 2010). If *D. melanogaster* sperm and seminal fluids are disrupted (or have higher levels of oxidative stress due to reduced levels of circulating melatonin) when males are exposed to chronic dim night lighting, this may reduce their capacity to successfully fertilise their mate's eggs and increase her oviposition rate. Further investigation into these potential mechanisms is warranted.

2.6 Conclusion

Overall, we have demonstrated a detrimental effect of chronic exposure to dim artificial light at night for two traits related to offspring production (the probability of a female commencing

oviposition and the number of eggs laid), and critically, a reduction in adult survival in *D. melanogaster*. This species is clearly capable of living in a wide variety of environments (Keller, 2007) making it an ideal species to assess the broad effects of artificial night lighting. However, our understanding of how (or indeed whether) other species, in particular invertebrates, are similarly resilient is currently limited. Given the importance of invertebrates for ecosystem health and function, and the likelihood that urban nights are set to become increasingly brighter, this knowledge will be critical for the effective management of our urban ecosystems and their future diversity.

Original paper

Chapter 3:

Dim artificial light at night affects mating, reproductive output and reactive oxygen species in *Drosophila melanogaster*

by

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3.1 Abstract

Humans are lighting the night-time environment with ever increasing extent and intensity, resulting in a variety of negative ecological effects in individuals and populations. Effects of light at night on reproductive fitness traits are demonstrated across taxa however, the mechanisms underlying these effects are largely untested. One possible mechanism is that light at night may result in perturbed reactive oxygen species (ROS) and oxidative stress levels. Here we reared *Drosophila melanogaster* under either dim (10 lx) light or no light (0 lx) at night for three generations and then compared mating and lifetime oviposition patterns. In a second experiment, we explored whether exposure to light at night treatments resulted in variation in ROS levels in the heads and ovaries of 6-, 23- and 36-day old females. We demonstrate that dim light at night affects mating and reproductive output: 10 lx flies courted for longer prior to mating, and female oviposition patterns differed to 0 lx females. ROS levels were lower in the ovaries but not heads, of 10 lx compared to 0 lx females. We suggest that reduced ROS levels may reflect changes in ovarian physiology and cell signalling which may be related to the differences observed in oviposition patterns. Taken together, our results indicate negative consequences for invertebrates under more stressful, urban, lit conditions and further investigation into the mechanisms driving these changes is warranted to manage invertebrate communities in a brighter future.

3.2 Introduction

The presence of artificial light at night (ALAN) is linked to species-wide shifts in behavioural and physiological traits, including courtship and mating, offspring production, growth and survival (Navara and Nelson, 2007, Longcore and Rich, 2004). These effects are evident even at relatively low levels of ALAN (≤ 30 lx), typical of the nocturnal light environment in some urban and peri-urban spaces. Field studies demonstrate a negative relationship between the presence of dim ALAN (≤ 5 lx) and reproductive success and juvenile growth in birds (Dominoni et al., 2013a, Dominoni et al., 2013c, Raap et al., 2016) and reproduction in mammals (LeTallec et al., 2015, Robert et al., 2015), while laboratory experiments in hamsters and rats link exposure to dim ALAN (between 1 and 5 lx) with increases in tumour growth rates (Blask et al., 2009) and immune suppression (Bedrosian et al., 2013). Similar responses to chronic exposure to dim ALAN (between 10 and 30 lx) are observed in invertebrates. Field experiments demonstrate lower mating success in moths (*Operophtera brumata*) (van Geffen et al., 2015b) and female aphids (*Megoura viciae*) were more likely to switch to an asexual mode of reproduction under dim ALAN conditions with potential implications for winter survival (Sanders et al., 2015). Furthermore, laboratory experiments exploring the effects of dim ALAN report decreased adult longevity and reduced oviposition and egg number of young *Drosophila melanogaster* females (McLay et al., 2017) and, a decline in the sexual attractiveness of female sex pheromones and accelerated juvenile development in the moth *Mammestra brassicae* (van Geffen et al., 2015a).

A potential driver of the observed physiological effects of dim ALAN is the possibility that ALAN also affects levels of reactive oxygen species (ROS). ROS are a suite of highly reactive by-products of the oxidation-reduction (redox) reactions that occur as part of normal cell metabolism (Dowling and Simmons, 2009). Low levels of ROS are essential for cell-signalling (Poli et al., 2004, Droge, 2002), however very high levels can cause oxidative stress, leading to damage to DNA, proteins and lipids and consequent effects on organism fitness (Metcalfe and Alonso-Alvarez, 2010). ROS is so critical to normal metabolic function that it is proposed as a major mechanistic driver for the process of aging and a suite of life history constraints (Dowling and Simmons, 2009, Harman, 1956). Accordingly, male and female fertility is adversely affected by both high (for reviews see Agarwal et al., 2012, Venkatesh et al., 2009),

and low levels of ROS and oxidative stress (de Lamirande et al., 1997). Gametes are particularly vulnerable to the negative effects of high ROS and oxidative damage to DNA can lead to transgenerational effects for offspring viability (Metcalfe and Alonso-Alvarez, 2010). Together, these observations suggest that ROS may be important mediators in the trade-off between reproduction and longevity (Dowling and Simmons, 2009, Metcalfe and Alonso-Alvarez, 2010).

Here, we investigate the potential of variation in ALAN (0 lx or 10 lx) to affect (i) reproductive output in the form of patterns of mating behaviour, lifetime oviposition and offspring development success and, in a second experiment, potential effects on physiology through (ii) differences in ROS levels of somatic (using the head) (Neretti et al., 2009) and reproductive tissue (ovaries) and accompanying morphological changes (measured through ovarian area). We use the model invertebrate *Drosophila melanogaster*, a species with demonstrable reduction in early reproductive effort and adult survival in the presence of 10 lx ALAN (McLay et al., 2017). We predict that flies subjected to the presence of ALAN during juvenile development and the adult stage of the life cycle will have perturbed ROS levels compared to flies reared with no light at night (0 lx). Furthermore, we expect a concomitant reduction in mating success, oviposition rates and offspring development.

3.3 Methods

3.3.1 Fly Stocks

A stock population of *Drosophila melanogaster* was created from 100 adult females and 50 adult males collected in April 2015 from Oak Ridge Winery in the Yarra Valley, Victoria, Australia (-37.686908, 145.457438). Stock flies were maintained on Bloomington's cornmeal medium (Brent and Oster, 1974) under standard conditions, at a density of approximately 50 male and 50 female flies for 34 generations, in a retrofitted incubator at 28 ± 1°C, prior to the start of the experiment (for further details, see McLay et al., 2017).

3.3.2 Light Treatments

We created two light treatments using Westinghouse incubators, as described by McLay et al. (2017). Both light treatments had an identical 12hr daytime lux (2600 lx - equivalent to an overcast day, 6800 kelvin), followed by one of two 12hr night time lux (either 0 lx, 0 kelvin or

10 lx, 5900 kelvin) ALAN treatments. To ensure no incubator bias, flies and their corresponding ALAN environment were rotated between three incubators every two to three days and were randomly positioned within each incubator.

3.3.3 Experimental Flies

To create our experimental generation of flies, we allocated five stock bottles of approximately 50 recently emerged female and male flies to the light (10 lx) and dark (0 lx) ALAN treatments. We maintained flies in their designated light environment for a further two generations in the same manner as reported previously (McLay et al., 2017), after which time we collected newly emerged (< 6 hours old) virgin female and male flies under mild CO_2 anaesthesia and transferred them to individual vials to mature for two days before pairing flies to mate. Measurement of reproductive output was conducted in two blocks (N = 66 male and female pairs from generation 35; N = 70 pairs from generation 36). We used data from this experiment and our previous study (McLay et al., 2017) to inform female age classes for the ROS study. The ROS assay and ovarian area study was also run over two blocks (N = 267 flies from generation 51; and N = 259 flies from generation 52).

3.3.4 Effect of ALAN on reproductive output

Mating behaviour - To assess whether mating behaviour varied under the two ALAN treatments, we paired 136 female and 136 male flies (N = 66 pairs in block 1, N = 70 pairs in block 2) in vials containing standard medium. Each pair was reared under the same light treatment but originated from different vials and thus were not siblings. A pair was permitted 30 minutes to commence mating. Flies that commenced copulation within the 30-minute time interval were allowed to complete copulation after which time the male was discarded; pairs that did not commence copulation within 30 minutes were also discarded. For each trial, the time until male wing extension, defined here as time to courtship (Cobb and Jallon, 1990), the time to copulation and the duration of copulation were recorded. To explore whether the number of eggs laid and success of development from egg to adult varied between the two light environments, we transferred a random subset of the mated females from the mating behaviour assay (N = 38 females, 31 from block 1; N = 40 from block 2) to individual vials containing fresh standard medium.

Oviposition patterns - We counted the total number of eggs laid by each of the females over a maximum of eight 24-hour time points (at 3, 9, 13, 16, 20, 27, 30 and 34 days) over their adult lives. At the start of each time point, the female was transferred to a vial with standard medium, dyed blue (Queen Fine Foods, Alderley, Australia) to increase egg visibility. After 24hr the female was returned to a new vial containing standard medium until the next time point. The final time point (day 34) was chosen to cover the median lifespan of *D. melanogaster* under ALAN conditions: our previous work demonstrates that approximately half of flies reared under 10 lx ALAN conditions are likely to have died by day 34 (McLay et al., 2017). After day 34, any surviving females were discarded, as by this time they would have almost certainly exhausted their store of sperm (Lefevre and Jonsson, 1962). The total number of eggs laid at each time point was counted twice by visual inspection under magnification.

Egg to adult success – To assess variation in the number of emerging adult offspring, we allowed eggs from the Day 3, 20 and 34 egg counts to develop to the adult stage. These time intervals represented different slopes in the survival curve of flies under these ALAN conditions (little to no mortality at Day 3, both 0 lx and 10 lx = 98% survival; a slow decline in survival to Day 20, 0 lx = 96% survival, 10 lx = 86% survival; and a sharp decline in survival to Day 34, 0 lx = 77% survival, 10 lx = 57% survival) (McLay et al., 2017). Vials were checked every one to two days until the first adult eclosed and then for a further seven days, after which time the vial was discarded. The number and sex of all emerging adults was recorded and the egg to adult success for each vial was determined as the number of emerging adults divided by the total number of eggs laid.

3.3.5 Effect of ALAN on ovarian area and comparative ROS levels in the head and ovary

In a second experiment, we investigated the effects of the different light regimens on ovarian area (as a proxy for the number of eggs remaining) and on ROS levels in heads and ovaries. A total of 526 single-mated females (N = 267 in block 1; N = 259 in block 2) were randomly allocated to one of three age cohorts (young = 6 days; medium-age = 23 days; old = 36 days post-eclosion). We used heads for somatic tissue ROS levels (Neretti et al., 2009) and ovaries to determine ROS levels in female germ cells. Once a female had reached her designated age, we lightly anaesthetised her with CO_2, removed the head and dissected out the ovaries.

Ovarian area – To explore whether exposure to ALAN resulted in differences in developing gametes, we used total ovarian area as a proxy for the number of eggs remaining when the female was assessed. This assumption is justified, as in our population ovarian area correlates with the number of visible eggs (stages 9 to 14) (Bate and Martinez Arias, 1993) (mean ± SE; total eggs: 26.65 ± 2.45, N = 20 females; $F = 111.1$, $R^2 = 0.86$, $P < 0.0001$) and mature eggs (stages 13 and 14) (5.65 ± 2.33, N = 20 females; $F = 49.97$, $R^2 = 0.74$, $P < 0.0001$). Ovaries were photographed at x 64 magnification with a dissecting microscope (Olympus SZX16, Tokyo, Japan), with attached camera (SONY ILCE-QX1, Tokyo, Japan). We calculated total ovarian area of each fly (mm^2) by drawing a line around the perimeter of each ovary using Image J software (NIH, Rockville, USA).

Comparative levels of ROS – To assess whether total ROS levels varied under the different light treatments at different female ages. We measured total ROS using a 2'7'-dichlorohydrofluorescin diacetate (H2DCFDA) assay, modified from Wang and Joseph (1999), in which the non-fluorescent DCFH is oxidised by ROS to form fluorescent DCF and can be detected by fluorometry (Wang and Joseph, 1999). From each light treatment and age cohort, samples of ovaries from single-mated flies (N = 50 pools of five pairs of ovaries per pool) and individual heads (N = 108 females) were assayed. Briefly, individual samples were placed into 50 μl of cold lysis buffer (TPER, Thermo Fisher Scientific, Rockford, USA) in a 96 well PCR plate (SSI, California, USA) on ice. We then homogenised each sample for 45 secs using a micro pestle made from a modified sterile 1000 μl pipette tip (Eppendorf, Hamburg, Germany). The plate was centrifuged at 400 x *g* for five minutes at $4^{O}C$ and 10 μl of supernatant transferred to a 96-well black assay plate (Greiner Bio-one, Neuberg, Germany). We added 40 μl of cold 10 mM solution of 2'7'-H2DCFDA (Sigma-Aldrich, Castle Hill, Australia) dissolved in dimethyl sulfoxide (Sigma-Aldrich, St Louis, USA). The plate was incubated in darkness for 45 minutes at $37^{o}C$ and then read for fluorescence in a microplate reader (PerkinElmer, EnSpire Multimode, Waltham, USA) at 485nm excitation and 535nm emission (Wang and Joseph, 1999). We commenced all assays between 2.5 - 3hr after incubator lights on; average time between fly death and the start of the assay was 2.0 ± 0.5hr. All samples were kept on ice until dissections and photographs were complete. For all ROS assay plates, we used positive/quality control and negative control samples. The positive/quality control was prepared separately and consisted of the supernatant from 200 whole flies from the stock population, homogenised in 3000 μl

TPER, and stored at -80^{o}C in sub-aliquots until required. The negative control contained all reagents except the tissue extract for each plate. Samples and controls were run in triplicate across plates and the mean fluorescence reading (subtracting the negative control) was used for analysis, where the coefficient of variation (CV) for the triplicate reading was < 15%. Inter-plate (CV of positive/quality controls) and intra-plate (CV of the mean CV of the first two and last two ovarian and head readings per plate) specific analyses were undertaken to assess the consistency of readings between plates.

3.3.6 Statistical analyses

Analyses were performed in R (R core team 2016) using the *lme4* software package (Bates et al., 2015). Prior to analysis, data were assessed for normality and transformed where appropriate (time to commencement of courtship and time from commencement of courtship to onset of copulation were log transformed, and time for copulation was cube root transformed). We used standard least squares linear models to explore differences in mating behaviours, total ovarian area and ROS levels; and a generalised linear model (GLM) with a binomial error distribution and logit link function to explore differences in the proportion of pairs commencing copulation in 30 minutes and numbers of females surviving to Day 34. Generalised linear mixed models (GLMM) fitted by maximum likelihood (ML) assuming a binomial error distribution and logit link function were used for all other data for reproductive output except for cumulative number of eggs, which did not include a binomial function and was fitted with restricted maximum likelihood (REML). We included light treatment and block as categorical factors in all models and other factors as appropriate: age cohort as a categorical factor in total ovarian area and ROS levels; maternal age as a continuous and polynomial variable in number of females laying at least one egg at any egg count and cumulative number of eggs laid per female; female identity as a random effect for all oviposition and offspring development models; number of days survived as a continuous variable for number of females laying at least one egg at any egg count; number of eggs laid per female at an egg count as a continuous variable for the proportion of eggs to emerge as adults where some adults emerged and for the sex ratio of offspring. Interactions between all these variables were included. The significance of parameters was assessed using hierarchical backwards stepwise deletion, dropping terms from the model (except the main parameter of interest, light treatment) where $P > 0.10$. *Post hoc* Tukey's tests were used to determine

differences between groups. Unless otherwise stated all data presented are means ± standard errors and the level of statistical significance was taken as $P < 0.05$.

3.4 Results

3.4.7 Effect of ALAN on reproductive output

Mating behaviour - Flies in the 10 lx treatment took longer from the onset of courtship to commence copulation than 0 lx flies ($P = 0.03$; Table 3-1c). In contrast, the probability of copulation commencing within 30 minutes; the time taken between first introduction into the vial and the onset of male courtship behaviour; and, the total duration of copulation, were comparable for the two light treatments (All $P > 0.15$; Table 3-1a-b, d).

Oviposition patterns and survival – Female survival to Day 34 ($\chi^2 = 0.42$, $P = 0.52$) and the number of females laying at least one egg at a given egg count ($P = 0.43$, Table 3-1e) were comparable for the two light treatment groups. Overall, the likelihood of an egg being laid varied with age ($P < 0.005$, Table 3-1e), but the relationship between maternal age and the probability of laying an egg was curvilinear ($P < 0.005$; Table 3-1e) and this pattern varied across the two ALAN treatments ($P = 0.01$; Table 3-1e; Figure 3-1a). A similar pattern was observed for the cumulative number of eggs laid per female. The cumulative number of eggs laid was comparable for 0 lx and 10 lx females ($P = 0.14$; Table 3-1f), but the relationship over time was non-linear ($P < 0.005$; Table 3-1f) and varied across the two ALAN treatments ($P < 0.005$; Table 3-1f, Figure 3-1b).

Egg to adult success – The probability that a vial with eggs present produced adult flies was the same for both light treatments ($P = 0.91$; Table 3-1g). However, no adults emerged from the Day 34 vials (number of vials with adults emerging for 0 lx Day 3 females = 33/36, Day 20 females = 5/22, Day 34 females = 0/0; 10 lx - Day 3 females = 28/32, Day 20 females = 3/19, Day 34 females = 0/0; $P < 0.005$; Table 3-1h). The proportion of eggs emerging as adults ($P = 0.37$; Table 3-1h) and adult sex ratio ($P = 0.97$, Table 3-1i) were comparable between the 0 and 10 lx treatments.

3.4.8 Effect of ALAN on ovarian area and comparative ROS levels in the head and ovary

Ovarian area and survival – There was no difference in ovarian area between the ALAN treatments (P = 0.75; Table 3-2a). The proportion of flies that survived to Day 34 was also comparable (χ^2 = 0.27, P = 0.61).

Comparative levels of ROS – The level of ROS in female's heads did not differ between the ALAN treatments (P = 0.33; Table 3-2b; Figure 3-2a), but 10 lx females had lower ovarian ROS levels compared to 0 lx females (P = 0.04; Table 3-2c; Figure 3-2a). ROS levels of both heads and ovaries varied with female age (heads: P < 0.005, and ovaries: P = 0.01 respectively, Table 3-2b-c, Figure 3-2b-c). *Post hoc* Tukey's tests revealed higher ROS levels in the heads of Day 23 compared with Day 36 flies, but no differences between Day 6 flies and either Day 23 or Day 36 flies. Additionally, ROS levels were higher in the ovaries of Day 6 compared with Day 36 flies but no different to flies of Day 23. The intra and inter-specific plate CVs were 14.91% and 10.50% respectively.

Table 3-1. Statistical models exploring the effect of artificial light at night (ALAN) on reproductive output in *D. melanogaster*. Besides the main variable of light treatment only those variables contributing to the minimal adequate model are reported. All statistics are mean ± standard error except where indicated.

Model Parameters	Mean ± SE or Proportions		Statistic, P value
	0 lx	10 lx	
Mating Behaviour			
a) Number of pairs commencing copulation in 30 minutes			
Light treatment	62/68	58/68	χ^2 = 1.14, P = 0.28
b) Time to commencement of courtship (log secs)			
Light treatment	4.60 ± 0.11	4.86 ± 0.14	$F_{1, 111}$ = 2.10, P = 0.15
c) Time from commencement of courting to onset of copulation (log secs)			
Light treatment	4.51 ± 0.16	5.02 ± 0.17	**$F_{1, 120}$ = 4.65, P = 0.03**
d) Time for copulation (cube root secs)			
Light treatment	9.60 ± 0.07	9.63 ± 0.07	$F_{1, 116}$ = 0.08, P = 0.78
Oviposition and offspring development			
e) Number of females laying at least one egg at any egg count			
Light treatment	185/249	132/199	χ^2 = 0.62, P = 0.43
Maternal age			**χ^2 = 28.40, P < 0.005**
Maternal age x Maternal age			**χ^2 = 17.45, P < 0.005**
Light treatment x (Maternal age x Maternal age)			**χ^2 = 6.89, P = 0.01**
f) Cumulative number of eggs laid per female			
Light treatment	81.25 ± 2.79	72.88 ± 3.12	χ^2 = 2.15, P = 0.14
Maternal age			**χ^2 = 127.31, P < 0.005**
Maternal age x Maternal age			**χ^2 = 15.78, P < 0.005**
Light treatment x (Maternal age x Maternal age)			**χ^2 = 12.79, P < 0.005**
g) Egg to adult success – proportion of vials where eggs were laid that had any adults emerge			
Light treatment	38/65	31/54	χ^2= 0.01, P = 0.91
Maternal age			**χ^2= 39.90, P < 0.005**
h) Egg to adult success – proportion of eggs to emerge as adults where any adults emerged			
Light treatment	0.51 ± 0.03	0.48 ± 0.03	χ^2 = 0.81 P = 0.37
i) Sex ratio of offspring (Days 3 and 20)			
Light treatment	0.50 ± 0.03	0.48 ± 0.03	χ^2 = 0.001 P = 0.97

Table 3-2. Statistical models exploring the effect of artificial light at night (ALAN) on reactive oxygen species (ROS) levels and ovarian area. a) Total ovarian area; b) ROS levels in female heads; and c) ROS levels of ovaries; Besides the main variable of light treatment only those variables contributing to the minimal adequate model are reported. All statistics are mean ± standard error.

Model Parameters	Mean ± SE		Statistic, P value
	0 lx	10 lx	
Ovarian area			
a) Total ovarian area			
Light treatment	1.24 ± 0.06	1.27 ± 0.06	$F_{1, 110} = 0.10$, P = 0.75
ROS			
b) ROS levels - female head			
Light treatment	234.76 ± 8.78	222.22 ± 9.93	$F_{1, 108} = 0.97$, P = 0.33
Female age			$F_{2, 108} = 5.64$, P < 0.005
c) ROS levels - ovaries			
Light treatment	111.07 ± 11.14	87.25 ± 6.88	$F_{1, 50} = 4.49$, P = 0.04
Female age			$F_{2, 50} = 5.05$, P = 0.01

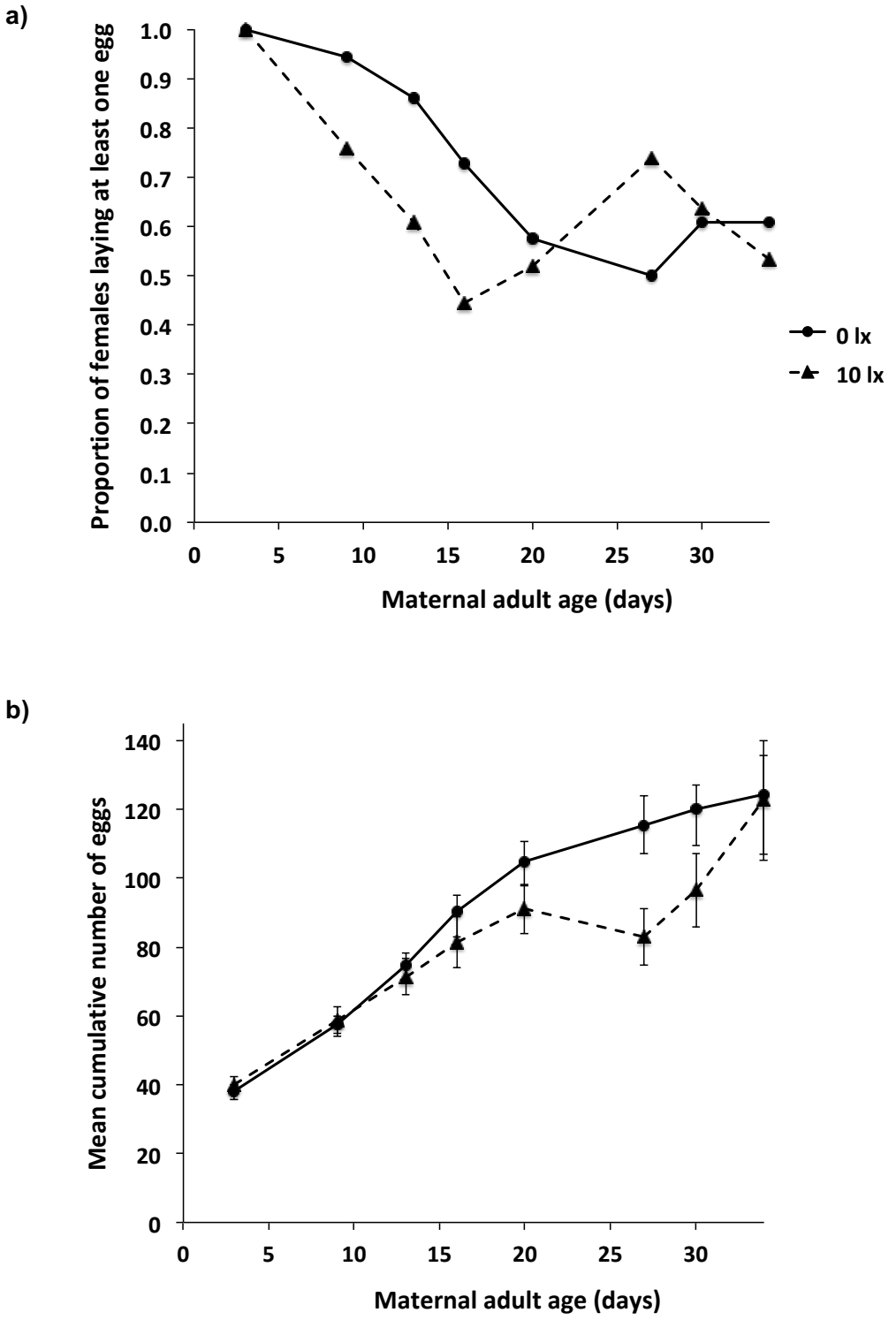


Figure 3-1. Oviposition varying with maternal age **a)** Proportion females ovipositing varying with maternal age (significant interaction term of treatment x maternal age x maternal age, P = 0.01 N = 348); and **b)** Mean cumulative number of eggs varying with maternal age (significant interaction term of treatment x maternal age x maternal age, P < 0.005, N = 305). Error bars represent SE.

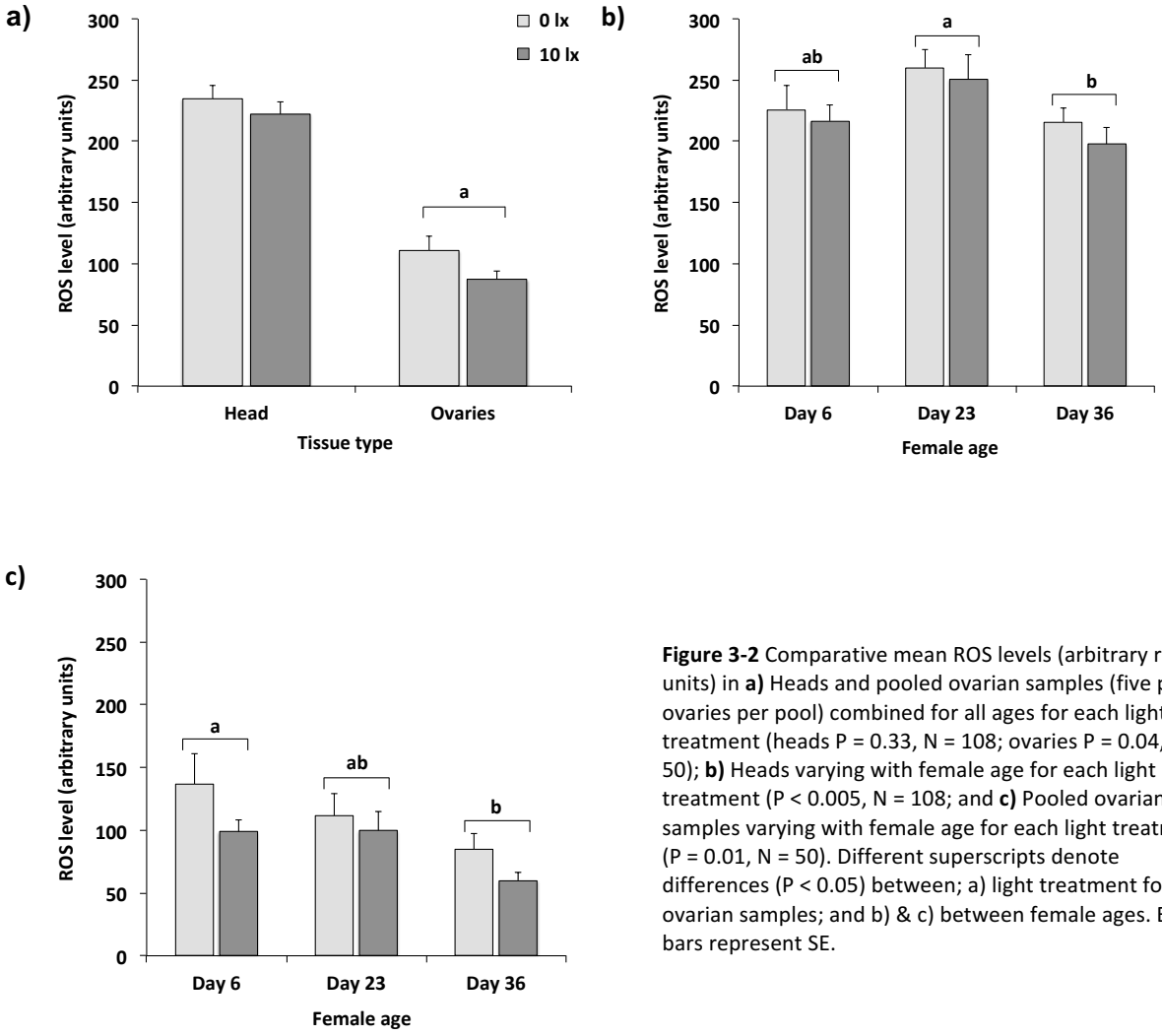


Figure 3-2 Comparative mean ROS levels (arbitrary relative units) in **a)** Heads and pooled ovarian samples (five pairs of ovaries per pool) combined for all ages for each light treatment (heads P = 0.33, N = 108; ovaries P = 0.04, N = 50); **b)** Heads varying with female age for each light treatment (P < 0.005, N = 108; and **c)** Pooled ovarian samples varying with female age for each light treatment (P = 0.01, N = 50). Different superscripts denote differences (P < 0.05) between; a) light treatment for ovarian samples; and b) & c) between female ages. Error bars represent SE.

3.5 Discussion

This study provides three key findings suggesting that exposure to dim ALAN has behavioural and physiological consequences. First, chronic exposure to dim ALAN of 10 lx was associated with an increase in the time taken between the onset of courtship and the commencement of copulation. Second, the pattern of oviposition over a female's life was different between light treatments, with both the likelihood of a female laying eggs within a 24-hour period and cumulative eggs laid over a female's life varying with the interaction between light treatment and maternal age. Finally, ROS levels of ovaries were comparatively lower in flies under 10 lx compared to the 0 lx treatment.

Our result that flies reared under 10 lx spent significantly longer courting but were equally likely to eventually copulate is one of the first experimental studies to record a difference in courting and mating behaviour under dim (10 lx) conditions. To our knowledge, these behaviours have yet to be assessed in vertebrates under dim ALAN. However, Botha et al. (2017) demonstrated interactions between light treatment and other variables in the duration of mating and number of mating bouts in crickets *(Teleogryllus commodus*). Additionally, a field study in moths (*Operophtera brumata*) demonstrated reduced mating success for females under 10 lx ALAN conditions (van Geffen et al., 2015b). Given the peak time for mating in *D. melanogaster* is during daylight hours (Sakai and Ishida, 2001), which is when our mating assays were conducted, it is unlikely that the presence of light during a trial is responsible for the behavioural differences observed here. Our study did not aim to identify possible mechanisms for this change in behaviour, and we note that van Geffen et al. (2015b) did not investigate courtship behaviour. However, the fact that a key aspect of mating behaviour shifted following lifetime exposure to ALAN suggests that, in both species, its presence has the potential to interfere with mating *per se*. In another species of moth (*Mamestra brassicae)*, ALAN is related to reduction in the attractiveness of female sex pheromones, which is likely to disrupt mating cues (van Geffen et al., 2015a). *D. melanogaster* have cuticular hydrocarbons that act as sex- and species-specific pheromone cues (Howard and Blomquist, 2005) and are subject to environmental perturbation (Higgie et al., 2000), but whether they exhibit similar variation under ALAN is currently untested and merits investigation. Regardless of the underlying mechanism, the increased time spent courting represents a potential fitness cost

for ALAN, due to increased competition and vulnerability to predators (Endler, 1987, Magnhagen, 1991).

Age-related declines in fecundity and oviposition rates are common across taxa (Clutton-Brock, 1988) including insects such as Coleoptera (Tanaka, 1990), Lepidoptera (Braby and Jones, 1995), Orthoptera (Carrière and Roff, 1995), Diptera (Jann and Ward, 1999), as well as *D. melanogaster* (Partridge et al., 1986) and such declines in propensity to lay and egg number over time were paralleled here. In contrast, while a reduction in the number of eggs produced under ALAN conditions in young adult females was previously reported for *D. melanogaster* (McLay et al., 2017), this is the first evidence for variation in patterns of lifetime egg production and variation in dim ALAN. The interaction we observed between female age and light treatment suggests that such an investigation was warranted, as the relationship was non-linear and thus, assessing variation within a single age-class may not be representative of lifetime oviposition. It is unlikely the observed pattern of oviposition was driven by differences in survival between the two light treatments, as the likelihood of survival to Day 34 was the same for both treatments and thus it seems that this represents a real difference in the pattern of offspring production. Maternal age effects aside, our study did not aim to disentangle male and female contributions to overall egg production. Male *D. melanogaster* transfer seminal fluids during mating, which stimulate egg production, ovulation and sperm retention (Wolfner, 2002, Gillott, 2003). Female *D. melanogaster* typically retain sperm for approximately 14 days following mating (Qazi et al., 2003) and offspring production is positively related to sperm storage (Qazi et al., 2003). There is the potential that the amount or quality of sperm and/or seminal fluids transferred during mating or subsequently stored, differed between the light treatments. Such a mechanism may explain why the oviposition curves initially diverge at approximately 15 days in the current study. Therefore, further investigations into the relative contributions of females and males to egg production is warranted.

A potential underlying mechanism for the observed physiological and behavioural effects of dim ALAN is its potential to suppress endogenous melatonin production (Blask et al., 2005, Blask et al., 2009, Brainard et al., 1982). The photosensitive indolamine melatonin is a key driver of circadian rhythm, as well as a powerful antioxidant (for review see Reiter et al., 2010). The primary site of its nocturnal production across taxa is in the head (the location of primary

photoreceptors) (Helfrich-Forster et al., 2001, Vivien-Roels and Pevet, 1993) including in *D. melanogaster* (Callebert et al., 1991, Finocchiaro et al., 1988) and its reduction can lead to circadian disruption and a change in levels of ROS (Pandi-Perumal et al., 2006) although this has not yet been demonstrated in *D. melanogaster.*

Our data did not support the oft-cited prediction that in the presence of ALAN, levels of ROS are likely to be increased (Tan et al., 2010, Jones et al., 2015, Pandi-Perumal et al., 2006, Reiter et al., 2003, Navara and Nelson, 2007). On the contrary, we found no difference in ROS levels in female heads between light treatments and comparatively lower levels of ROS in the ovaries of 10 lx females compared to their 0 lx counterparts. In the absence of an effect of ALAN on total ovarian area or on the cumulative number of eggs laid, it is unlikely that the difference between the two light treatments arises due to reduction in the quantity of material assayed and thus a reduced signal. Instead, we suggest that the observed variation reflects ALAN-induced changes in ovarian physiology that may explain differences in the pattern of eggs laid over a female's lifespan. A certain level of ROS is required for signalling pathways in cells (Droge, 2002). In *D. melanogaster*, ROS generated from nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) is required for normal ovulation (Ritsick et al., 2007) and it is conceivable that these pathways are modulated in the presence of ALAN. The implications of this effect on fitness is currently unclear, as aside from changes for the ALAN exposed females in the oviposition pattern and cumulative egg number over time, the egg to adult success rates of offspring were unchanged in the current laboratory study.

Intriguingly, despite detectable differences in the ovaries between the two light treatments, ROS levels in the heads did not differ. This highlights the possibility of tissue-specific responses to ALAN arising through variation in the levels and pattern of ROS generation between the two tissue types. Ovaries contain rapidly dividing cells that typically contain higher numbers of mitochondria than somatic cells (Cree et al., 2015) and as ROS generation occurs primarily in mitochondria (Finkel and Holbrook, 2000), we may expect ovaries to generate greater amounts of ROS than somatic tissue. A direct link between metabolic rate and ROS levels remains controversial (Salin et al., 2015, Alonso-Alvarez et al., 2017), nonetheless, extra-mitochondrial ROS production, for example from NOX in reproductive tissues (Alonso-Alvarez et al., 2017, Ritsick et al., 2007) could also lead to discernible differences between tissues under ALAN conditions. Variation in these two ROS generators could also explain the age-

specific differences in ROS levels in both heads and ovaries between age cohorts (higher ROS levels in the Day 6 cohort compared to the Day 36 cohort). Higher metabolic activity and investment in reproduction is associated with young organisms, while older individuals undergo senescence (Frisard et al., 2007, Sohal and Weindruch, 1996), so we may expect to see shifts in ROS levels between the beginning and end of adult lifespan.

Our current study measured overall ovarian ROS at a specific timepoint (during the daylight and not during the dark period) and was not designed to identify specific signalling pathways. We note that ROS levels vary in different tissues over a 24hr period due to rhythms in circadian activity and antioxidant production (Hardeland et al., 2003). Therefore, we do not know whether the observed ROS levels reflect the overall diurnal changes in redox status of the ovary. Future experimentation with more sampling time points is needed to assess relative temporal, tissue-specific differences in ROS. Additionally, our experiment was undertaken in flies that had been under light treatment for three generations, it is possible (albeit not tested) that only flies with inherently lower levels of ROS progressed to the third generation. Further multigenerational studies would elucidate whether selection is occurring between generations under ALAN. Moreover, this experiment was conducted in a benign laboratory environment, with constant temperature and food *ad libitum*. While the imposed light stress was strong enough to generate detectable differences within the ovaries it may not reflect the more extreme ecological stresses imposed in nature.

In conclusion, we have demonstrated that dim anthropogenic light at night has a detrimental impact on two traits related to reproductive fitness in *D. melanogaster* (mating behaviour and oviposition patterns over a female's life). Additionally, dim ALAN lowers ovarian ROS levels compared to a no light at night treatment, which may reflect altered ovarian physiology i.e. cell signalling. Further studies on effects of ALAN on mating cues and nocturnal/diurnal ROS loading are required to understand the underlying mechanism(s) behind and consequences of our results. Given that we are lighting the world with increasing extent and intensity (Kyba et al., 2017), understanding the mechanisms behind and the consequences of this anthropogenically induced pollutant to populations and ecosystems is critical for the effective management of our urban ecosystems and their future diversity.

Original paper

Chapter 4:

Can artificial light at night drive local adaptation in *Drosophila melanogaster*?

by

Lucy Katherine McLay, Mark Philip Green and Therésa Melanie Jones

This work is currently being prepared for publication

4.1 Abstract

Artificial light at night (ALAN) is increasingly affecting our global landscape, with negative phenotypic effects on key fitness traits recorded across taxa. Consequently, ALAN has the potential to act as a selective agent to drive evolutionary change. However, currently studies testing this theory, excluding other urban stressors, are lacking. Here, we used experimental evolution to assess the potential for chronic ALAN to act as a selective agent driving genetic change in the model organism, *Drosophila melanogaster*. We reared five replicate populations of *D. melanogaster* in the laboratory under each of 0 lx or 10 lx at night over 25 generations and, following a reciprocal transplant experiment for two generations, we compared mating behaviour, oviposition, longevity (generation 17) and size and oxidative DNA damage (generation 27). We demonstrate that ALAN induced limited local adaptation in mating propensity (probability of mating), however other life history traits (oviposition, longevity, and size) and relative oxidative DNA damage showed no evidence of adaptive evolution. While we found weak evidence for ALAN to act as a driver of evolutionary change, we confirm short-term plastic phenotypic responses of flies reared under light at night: such as disrupted mating behaviour, lower fecundity and reduced longevity in females. Flies reared under ALAN were also smaller and females exhibited lower levels of oxidative DNA damage, the latter result aligning with our previous finding of reductions in ROS in ovaries under ALAN. While potentially limited in its impact under relatively benign laboratory conditions, our data demonstrate that even in the absence of other stressors, ALAN can act as a selective agent promoting evolutionary change. Whether the strength of selection increases under more natural stressful urban environments and ALAN's relative importance as an urban stressor remain to be determined.

4.2 Introduction

One of the most rapid and globally pervasive anthropogenic changes to the environment is the increasing extent and intensity of artificial light at night (ALAN) (Kyba et al., 2017). ALAN is linked to a range of changes in physiology and behaviour across a wide range of animal taxa, with demonstrable phenotypic effects on key fitness traits such as reproduction and survival (for reviews see Navara and Nelson, 2007, Gaston et al., 2014, Russart and Nelson, 2018, Desouhant et al., 2019). This phenotypic response indicates that organisms have enough phenotypic plasticity to survive under ALAN in the short-term, however, whether ALAN can act as a selective agent to promote long-term genetic change is untested (Hopkins et al., 2018). In general, the potential for urban stressors to act as a selective pressure that induces evolutionary change is well documented (Swaddle et al., 2015, Johnson and Munshi-South, 2017, Hopkins et al., 2018). Moreover, heritable variation in some phenotypic traits related to the presence of light at night are proposed (decreased phototaxis in moths originating from light, compared to dark at night areas: Altermatt and Ebert, 2016). However, to date, no study has determined whether this is the case with the exclusion of other stressors. Determining ALAN's potential as a driver of evolutionary change is achievable using an experimental evolution approach.

Experimental evolution is a powerful approach to compare traits across populations evolved under different specific imposed environmental parameters (Kawecki et al., 2012). Common approaches to experimental evolution include: common-garden experiments, where following a period of evolution, populations are reared in a common environment for one or more generations before assessment (Kawecki et al., 2012); and, reciprocal transplant experiments, where evolved populations are transplanted to the alternate environment for one or more generation prior to assessment (Blanquart et al., 2013). For logistic reasons, experimental evolution uses organisms with rapid generation times (Kawecki et al., 2012) and has been used to explore adaptation to environmental stressors such as temperature (Bennett and Lenski, 1993), nutritional stress (Kolss et al., 2009), saline environments (Dhar et al., 2011), competition (Santos et al., 1997, terHorst, 2011, Shenoi et al., 2016), predation risk (Reznick et al., 1997) and mating systems (Hollis and Kawecki, 2014, Hollis et al., 2019).

There are several ways that ALAN could generate local adaptation. The most likely mechanisms underpinning rapid adaptive change in urban environments are epigenetic modifications (Banta and Richards, 2018), regulation of gene flow (Hopkins et al., 2018) and selection on standing levels of genetic variation in a population (for review see Barrett and Schluter, 2008). ALAN may also induce evolutionary change by creating novel genetic variation in populations via mutagenesis (Hopkins et al., 2018). One method by which this could occur is via oxidative DNA damage (Jena, 2012), which is predicted to be higher for populations exposed to ALAN, due to ALAN-induced decreases in the antioxidant, melatonin, and resulting declines in endogenous antioxidant capacity (Reiter, 2002). Oxidative damage occurs when levels of reactive oxygen species (ROS), which are produced as a by-product of normal cell metabolism, get out of balance with an organism's antioxidant capacity (Dowling and Simmons, 2009). A certain level of ROS is necessary for cell-signalling (Droge, 2002), however high ROS levels can lead to DNA damage (Reiter et al., 2000, Metcalfe and Alonso-Alvarez, 2010) and potentially novel mutations (Cooke et al., 2003, Jena, 2012). To protect against this, organisms have evolved several endogenous antioxidants, such as melatonin, that can be upregulated when ROS levels begin to accumulate (Monaghan et al., 2009).

Here, we used the model organism *Drosophila melanogaster* to explore the potential for chronic exposure to ALAN to act as a selective agent for local adaptation after 15 and 25 generations. *Drosophila melanogaster* is commonly used for experimental evolution studies (for reviews see Burke and Rose, 2009, Kawecki et al., 2012) and is an ideal organism to explore local adaptation due to its short generation time and speed with which it can adapt (< 10 generations) (Rose et al., 1992, Blows and Hoffmann, 1993, Hoffmann and Parsons, 1993). Additionally, our previous studies using an ALAN experimental paradigm have demonstrated responses in key life history traits: decreased longevity, fecundity, altered mating patterns and decreased ROS levels following exposure to ALAN of 10 lx (McLay et al., 2017, McLay et al., 2018). To confirm the robustness of our previously reported short-term ALAN related shifts in life history traits, we also took baseline measures within the first two generations of the experimental period (thus assessing phenotype while controlling for maternal effects; Kawecki et al., 2012). Following a period of experimental evolution, we used a reciprocal transplant design for two generations to compare key life history traits (mating behaviour, fecundity, longevity and size), as well as the resulting degree of DNA damage in five replicate populations

of flies evolved under either 10 lx at night (10 lx ALAN treatment) or a dark, 0 lx night (0 lx ALAN treatment) for 15 (mating, fecundity and longevity) and 25 generations (oxidative damage and size). We predicted that ALAN would negatively affect fitness traits in the short term, confirming its potential as a selective agent, and that following a period of evolution under ALAN, life history traits and oxidative damage would demonstrate local adaptation.

4.3 Methods

4.3.1 Fly stocks

Experimental *Drosophila melanogaster* originated from wild caught adult flies (100 females, 50 males) from the Yarra Valley in Victoria, Australia (map reference: -37.686908, 145.457438). Prior to the experiment, the population had been maintained as five replicate lines (50 females and 50 males per population) for > 80 generations under standard conditions (McLay et al., 2017).

4.3.2 ALAN treatments

To assess whether ALAN can act as a selection pressure to promote local adaptation, we created two ALAN light treatments using retrofitted incubators (see McLay et al., 2017). Both treatments were exposed to 12hr of day (2600 lx; 6800 k; approximately equivalent to an overcast day); night-time light levels were either 12 hr of 0 lx, 0 k (0 lx treatment) or 10 lx, 5900k (10 lx treatment, approximately equivalent to a suburban street).

4.3.3 Experimental flies

At the start of the experiment, we created ten populations of 50 female and 50 male recently emerged flies (sourcing two replicate populations from each of our five stock populations) and allocated them equally to the two light treatments. We maintained these ten founding populations in their allocated ALAN environment under standard culturing conditions for 25 generations. At each generation, we randomly selected 50 female and 50 male recently emerged flies (approximately two days old) from each population to mate and oviposit for two to three days to contribute to the next generation, before discarding the adults so there were no overlapping generations. To ensure no incubator bias, we rotated populations between

four incubators every two to three days (maintaining ALAN treatment) and randomly positioned populations within each incubator.

4.3.4 Baseline assessment of life history traits

To determine phenotypic plastic responses following exposure to light at night we measured key life history traits (mating, oviposition and longevity), at evolved generation two (F_2) (thus minimising potential effects of maternal stock population). To assess variation in these traits, we transferred 30 individual virgin F_1 male and female pairs (pairs originated from the same ALAN treatment, but different population as per our established experimental paradigm for investigating the effects of ALAN (McLay et al., 2017, McLay et al., 2018)) to a clean glass vial (102 mm height x 15 mm diameter) containing standard medium (7 ml). Pairs were left for three days after which time they were transferred to a new vial for a further 24 h. Adults were subsequently discarded and vials left for offspring to develop. Newly eclosed (within 6 h) F_2 virgin male and female flies were collected and transferred individually to a clean standard food vial for six days. After six days we assayed mating behaviour (McLay et al., 2018), oviposition and longevity (McLay et al., 2017) (see Supplementary Table 1 at the end of Chapter 4 for sample sizes and Supplementary Figure 1 for experimental design).

Mating behaviour – To establish baseline mating behaviour for our populations and assess whether ALAN treatment affected mating parameters, individual female and male flies from different parental vials but the same treatment, were paired without the application of anaesthesia in vials containing standard medium and were left for 30 min to commence copulation (sufficient for 85% to copulate, McLay et al., 2018). For each trial, whether or not pairs copulated (defined here as mating propensity,) the time to courtship (defined as the time from the start of the trial until the first male wing extension), the time to copulation and the duration of copulation were recorded (McLay et al., 2018). Any pairs that did not commence copulation within the 30-min time interval were discarded. Mating assays were conducted between three and six hours after incubator dawn (the peak time for mating in *D. melanogaster*, Sakai and Ishida, 2001)) and the order of assessment with respect to ALAN treatment was randomised.

Fecundity and Longevity - Following the mating behaviour assay, individual males were immediately transferred to a fresh vial of standard medium and then subsequently transferred to a new vial every three to four days until death. To measure female fecundity and longevity, mated females were transferred individually to a fresh oviposition vial and left for 18 h for oviposition. To increase egg visibility (McLay et al., 2018) we used a dyed standard medium for this assay (0.00025% blue dye: nutrient medium; (Queen Fine Foods, Alderley, Australia)). The number of eggs laid by each female were counted twice by visual inspection under x25 magnification (Nikon dissecting microscope 122652, Melville, NY, USA) (McLay et al., 2018). Each female was subsequently transferred to a fresh vial every three to four days and monitored daily until death (as for males).

4.3.5 Assessment of life history traits in evolved populations

We tested for local adaptation of life history traits in relation to ALAN following a two-generation reciprocal transplant experiment to eliminate maternal effects (Kawecki and Ebert, 2004) at generation 17 (F_{17}) for mating behaviour, oviposition and longevity, and generation 27 (F_{27}) for wing size. For the reciprocal transplant phases, we created two adult bottles per population (each containing 50 males and 50 females) and allocated one to each of the two ALAN treatments resulting in 20 adult populations that varied in their evolved and current ALAN treatments. After 24h, adults were discarded and offspring were permitted to mature to adulthood. We paired 30 newly eclosed F_{16} virgin adults from each of the four experimental treatments with a member of the opposite sex originating from a different population, but same treatment in glass vials (*sensu* McLay et al., 2018). Pairs were allowed to mate and mature for three days, males were discarded, females were left for a further 24 h to oviposit and were then also discarded (as per F_2). F_{17} offspring were reared to adult, allowed to mature for six days and then assayed for mating success, oviposition and longevity (as for the F_2 generation above). In order to obtain sufficient replicates, this protocol for life history trait assessment at generation F_{17} was repeated, using eggs laid by the same females one consecutive day apart (thus maintaining the generational pedigrees) to create two blocks that was controlled for in analysis (see Supplementary Table 1 for sample sizes).

After a further 10 generations of evolution in our 10 lx and 0 lx ALAN populations, we tested whether ALAN treatment could promote adaptation in size (F_{27}) and in levels of oxidative DNA

damage (see below). The protocol from F_{25} to F_{26} was identical as for F_{15} to F_{16} (above) but to produce enough flies for DNA damage assessment, we used bottles (rather than vials) containing more flies for F_{26} to F_{27} than for than for F_{16} to $F_{17.}$ Thus, we transferred 10 newly emerged males and females from each F_{26} bottle into five new bottles (50 females and 50 males per bottle) and permitted them to mate and mature for three days, after which, we moved flies to new medium bottles and allowed females to oviposit for 24 h, before discarding adults and permitting the F_{27} generation to mature to adult. On the first day of eclosion, we subsequently took a random sample of approximately 32 each of newly eclosed males and females ensuring that each experimental treatment and bottle was equally represented. Flies were placed in 80% ethanol until wing size measurements were undertaken (following McLay et al 2017). See Supplementary Table 1 for sample sizes.

4.3.6 Assessment of variation in oxidative DNA damage

To test whether evolved ALAN populations differed in levels of oxidative DNA damage, F_{27} generation adults were maintained in their respective bottles for three days to mate and mature, after which time females and males were transferred in groups of 50 to single-sex bottles (maintaining their ALAN treatment) for a further 19 days. To reduce interference between developing larvae and female longevity, females were tipped into new medium every two days; males were maintained in their bottles for four days. To reduce potential gut contamination from the yeast contained in the medium, at day 19 we transferred flies to a clean bottle in the absence of food but with cottonwool soaked in 0.05 M sucrose solution for 24 h. After this time, the 20-day old flies were snap frozen in liquid nitrogen and stored in single sex same treatment (mixed population) groups of 30 at -80^{o}C until DNA damage assays were undertaken. See Supplementary Table 1 for samples sizes. We chose 20 days as our time period, as this is the age under our well-defined experimental paradigm, when differences in survival between ALAN treatments are evident but > 80% of flies were likely to be alive (McLay et al., 2017).

We used a well-established commercial assay to assess oxidative DNA damage (EpiQuik 8-OHdG DNA Damage Quantification Direct Kit (EpiGentek, Farmingdale, NY, USA)) (Valavanidis et al., 2009). We extracted DNA from pooled samples across treatment of 30 whole same sex flies using DNAzol Reagent (ThermoFisher Scientific, Waltham, MA, USA), as per product

instructions. DNA concentration and quality were measured using a NanoDrop One (ThermoFisher Scientific, Waltham, MA, USA) and samples with an A260/A280 ratio of absorbance > 1.8 (Wilfinger et al., 1997) were diluted to 100 ng/μl of DNA with RNA-free water (Qiagen, Hilden, Germany). 8-OHdG was measured using the EpiQuik 8-OHdG DNA Damage Quantification Direct Kit as per kit instructions. Briefly, 300 ng of the diluted DNA (3 μl) was added per sample well to the EpiQuik microplates. Female samples and controls were run in triplicate and male samples in duplicate across each plate. We used a negative control and standard curve as per kit instructions and absorbance was read at 450 nm using a microplate reader (Thermo Scientific Mulitskan EX, Waltham, MA, USA). The mean absolute 8-OHdG for each sample was used in the analysis, where the coefficient of variation (CV) for the repeated readings was < 15%. Inter-plate (mean of CVs of positive controls across plates) and intra-plate (average of mean of CVs for each plate) were undertaken to assess the consistency of readings between and within plates. The intra- and inter-plate CVs across all plates (both sexes) were 8.63% and 13.22%, respectively. See Supplementary Table 1 for sample sizes.

4.3.7 Statistical Analyses

Analyses were performed in R (R Core Team, 2016) using the *lme4* (Bates et al., 2015) and *coxph* (Therneau and Lumley, 2018) software packages. Prior to analysis, data were assessed for normality and transformed where appropriate (time to commencement of courtship and time from commencement of courtship to onset of copulation were cube root transformed, as per Chapter 4.3.1 in Quinn and Keough (2002)). For oxidative damage analysis, males and females were analysed separately because husbandry procedures differed between sexes. We used generalised linear mixed effects models (GLMER) with a binomial error distribution and logit link function to explore differences in the proportion of pairs commencing copulation in 30 min and the probability of a female commencing oviposition. We used standard least squares linear models to explore differences in mating behaviours, the number of eggs laid by ovipositing females, wing size and oxidative damage. Cox's proportional hazard regressions model was used for analysis of longevity data. For all models, we used ALAN treatment (either evolved and/or current ALAN treatment), block (where relevant) and their interactions as fixed categorical variables. Block was entered as a random variable in all relevant final models as preliminary models revealed no interaction between block and the other variables. Number of eggs laid was included as a continuous variable in female longevity analysis. The significance of

parameters was assessed using hierarchical backwards stepwise deletion, dropping terms from the model (except the main parameters of interest, ALAN treatment at F_2, evolved ALAN treatment and current ALAN treatment and the interaction of evolved and current ALAN treatment at F_{17} and F_{27}) where $P > 0.1$. Once the minimal model was obtained, excluded terms were reintroduced to confirm the model fit did not improve with their inclusion. Post hoc Tukey's tests were used to determine differences between groups. P values were adjusted using p.adjust (FDR function) (Benjamini and Hochberg, 1995) in R, to take into account multiple comparisons where families of statistical testing had more than three comparisons (Mating behaviour at F_2 and F_{17}). The level of statistical significance was taken as $P < 0.05$.

4.4 Results

4.4.8 Baseline assessment of life history traits (F_2)

The results in our baseline assessment of life history traits largely concur with our previous studies demonstrating phenotypic shifts in our populations with short-term exposure to ALAN (McLay et al., 2017, McLay et al., 2018).

Mating behaviour – Flies exposed to two generations of 10 lx ALAN took longer between the onset of courtship and the start of copulation ($P = 0.04$, Table 4-1c) and copulated for longer ($P = 0.04$, Table 4-1d) than flies exposed to two generations of 0 lx. The likelihood of flies to commence copulating within 30 min ($P = 0.07$, Table 4-1a) and time to commencement of courtship was comparable for 0 lx and 10 lx flies ($P = 0.80$, Table 4-1b).

Oviposition – The number of successfully mated females that laid at least one egg did not differ between ALAN treatments ($P = 0.69$, Table 4-1e), however, considering ovipositing females only, 10 lx females laid fewer eggs compared to 0 lx females ($P = 0.01$, Table 4-1f).

Longevity – Female, but not male, longevity was reduced when exposed to 10 lx compared to 0 lx ($P < 0.001$ and $P = 0.64$ respectively, Table 4-1g).

4.4.9 Assessment of life history traits in evolved populations (F_{17} and F_{27})

Our results in differences in life history traits after evolution under ALAN, showed little evidence that ALAN acts as a strong selection pressure promoting evolutionary change, although local adaptation was demonstrated for the probability of mating.

Mating behaviour – The likelihood of successful copulation within 30 min varied with the interaction between current and evolved ALAN treatment (P = 0.04, Table 4-2a, Figure 4-1a): flies evolved under 0 lx at night were less likely to mate when subsequently exposed to 10 lx at night compared to flies that remained in 0 lx but all other treatment groups were comparable. There was no effect of evolved ALAN treatment on the time to commencement of courtship (P = 0.48, Table 4-2b, Figure 4-1b). However, current ALAN treatment did affect time to courtship (P = 0.002, Table 4-2b, Figure 4-1b): flies currently exposed to 10 lx took longer to begin courting than those exposed to 0 lx. These patterns were consistent regardless of the ALAN environment they evolved under (interaction between current and evolved ALAN treatments: P = 0.22, Table 4-2b). Other measures of mating behaviour; time from courtship to copulation (Table 4-2c, Figure 4-2a) and time taken for copulation (Table 4-2d, Figure 4-2b) were comparable regardless of evolved or current ALAN environment.

Oviposition – The likelihood of females commencing oviposition was related to both their current (P = 0.01, Table 4-2e, Figure 4-3a) and evolved ALAN treatment (P < 0.001, Table 4-2e, Figure 4-3a) but not the interaction between current and evolved ALAN treatment (P = 0.08, Table 4-2e, Figure 4-3a), with females less likely to oviposit under 10 lx at any time. Additionally, evolved 10 lx females laid fewer eggs than evolved 0 lx females (P = 0.001, Table 4-2f, Figure 4-3b) and females currently exposed to 10 lx laid fewer eggs than females exposed to 0 lx (P = 0.03 Table 4-2f, Figure 4-3b). There was no interaction between ALAN treatment at F_{17} and evolved ALAN treatment in number of eggs laid (P = 0.46, Table 4-2f, Figure 4-3b).

Longevity – Female adult longevity was related to current and evolved ALAN treatment (P = 0.03 for both main effects, Table 4-3a, Figure 4-5a) but not their interaction (P = 0.60, Table 4-3a). Females evolved under and currently exposed to 10 lx died sooner than their 0 lx counterparts. In contrast, male longevity was comparable for all treatments (Table 4-3a, Figure 4-5b).

Wing length – Fly size varied with current ALAN treatment ($P < 0.001$, Table 4-3b), evolved ALAN treatment ($P = 0.01$, Table 4-3b) and sex ($P < 0.001$, Table 4-3b). Flies were smaller under 10 lx at F_{27} than their 0 lx counterparts, regardless of evolved ALAN treatment, and larger if they had evolved under 10 lx irrespective of their current treatment (Table 4-3b, Figure 4-4). There was no interaction between current and evolved ALAN treatment at F_{27} ($P = 0.16$, Table 4-3b, Figure 4-4). Females flies were larger than males ($P < 0.001$, Table 4-3b).

4.4.10 Assessment of variation in oxidative DNA damage (F_{27})

Oxidative DNA damage detected in females at generation F_{27} was related to their current ALAN treatment (females exposed to 10 lx had lower 8-OHdG levels than females exposed to 0 lx: $P = 0.01$, Table 4-4, Figure 4-6a) but not their evolved ALAN treatment ($P = 0.93$, Table 4-4, Figure 4-6a) or their interaction ($P = 0.55$, Table 4-4). In contrast, for males, oxidative damage was comparable regardless of current ($P = 0.40$, Table 4-4, Figure 4-6b), or evolved ALAN treatment ($P = 0.46$, Table 4-4, Figure 4-6b).

Table 4-1. Statistical models exploring the effect of ALAN exposure on the baseline (F_2) assessment of life history traits of mating behaviour, oviposition and longevity in *D. melanogaster*. Besides the main variable of ALAN treatment, only those variables contributing to the minimal adequate model are reported. All averages are reported from untransformed data as the mean ± standard error, proportions or median (interquartile range). * Analysis performed on cube root transformed data. #P value is FDR adjusted.

Model Parameters	Mean ± SE, Proportions or Median (interquartile range)		df	Statistic, P value
ALAN Treatment	**0 lx**	**10 lx**		
Mating behaviour				
a) Number of pairs commencing copulation within 30 minutes				
ALAN treatment	46/58 (79%)	38/60 (63%)	1	$\chi^2 = 3.71$, P = 0.07#
*b) Time to commencement of courtship (secs)**				
ALAN treatment	300 (120-534)	240 (120 – 630)	1	$F_{1, 104} = 0.07$, P = 0.80#
*c) Time from courting to copulation (secs)**				
ALAN treatment	60 (0-360)	180 (60-1005)	1	**$F_{1, 84} = 5.82$, P = 0.04#**
d) Copulation duration (secs) (F2 generation only)				
ALAN treatment	1050 (840-1260)	1190 (1080-1320)	1	**$F_{1, 84} = 5.58$, P = 0.04#**
Oviposition				
e) Number of females commencing oviposition				
ALAN treatment	43/45 (96%)	35/36 (97%)	1	$\chi^2 = 0.16$, P = 0.69
f) Number of eggs per female if any eggs laid				
ALAN treatment	26.58 ± 1.98	19.26 ± 1.59	1	**$F_{1, 78} = 7.78$, P = 0.01**
Longevity				
g) Adult longevity (days)				
Females				
ALAN treatment	39 (37-43)	33 (31-38)	1	**$\chi^2 = 14.39$, P < 0.001**
Males				
ALAN treatment	39 (37-43)	37 (31-43)	1	$\chi^2 = 0.22$, P = 0.64

Table 4-2. Statistical models exploring the potential for local adaptation to ALAN (interaction between current treatment at F_{17} or F_{27} and evolved treatment) in life history traits of mating behaviour and oviposition in *D. melanogaster*. Besides the main variable of current and evolved ALAN treatment and their interaction, only those variables contributing to the minimal adequate model are reported. All averages are reported from untransformed data as the mean ± standard error, proportions or median (interquartile range). * Analysis performed on cube root transformed data. #P value is FDR adjusted.

Model Parameters	**Mean ± SE, Proportions or Median (interquartile range)**		**df**	**Statistic, P value**
ALAN Treatment	**0 lx**	**10 lx**		
Mating behaviour (F_{17})				
a) Number of pairs commencing copulation within 30 min				
Current ALAN treatment	101/120 (84%)	77/120 (64%)	1	**χ^2 = 14.98, P = 0.001**#
Evolved ALAN treatment	88/120 (73%)	90/120 (75%)	1	χ^2 = 2.98, P = 0.22#
Current ALAN treatment x Evolved ALAN treatment			1	**χ^2 = 5.81, P = 0.04**#
*b) Time to commencement of courtship (secs)**				
Current ALAN treatment	227 (101-457)	361 (182-745)	1	**$F_{1, 221}$ = 12.32, P = 0.002**#
Evolved ALAN treatment	308 (127-587)	257 (131-686)	1	$F_{1, 221}$ = 0.98, P = 0.48#
Current ALAN treatment x Evolved ALAN treatment			1	$F_{1, 221}$ = 2.82, P = 0.22#
*c) Time from courtship to copulation (secs)**				
Current ALAN treatment	220 (36-650)	153 (42-606)	1	$F_{1, 178}$ = 2.32, P = 0.26#
Evolved ALAN treatment	208 (38-620)	211 (39-619)	1	$F_{1, 178}$ = 0.35, P = 0.66#
Current ALAN treatment x Evolved ALAN treatment			1	$F_{1, 178}$ = 0.98, P = 0.48#
d) Copulation duration (secs)				
Current ALAN treatment	1189.19 ± 26.07	1234.06 ± 29.96	1	$F_{1, 178}$ = 0.06, P = 0.80#
Evolved ALAN treatment	1183.36 ± 27.60	1233.28 ± 27.96	1	$F_{1, 178}$ = 0.22, P = 0.70#
Current ALAN treatment x Evolved ALAN treatment			1	$F_{1, 178}$ = 0.40, P = 0.66#
Oviposition (F_{17})				
e) Number of females commencing oviposition				
Current ALAN treatment	77/96 (80%)	41/75 (42%)	1	**χ^2= 7.70, P = 0.01**
Evolved ALAN treatment	70/85 (82%)	48/86 (56%)	1	**χ^2= 11.95, P < 0.001**
Current ALAN treatment x Evolved ALAN treatment			1	χ^2= 3.05, P = 0.08
f) Number of eggs per female if any eggs laid				
Current ALAN treatment	19.06 ± 1.65	14.37 ± 1.49	1	**$F_{1, 118}$ = 4.67, P = 0.03**
Evolved ALAN treatment	20.63 ± 1.70	12.77 ± 1.40	1	**$F_{1, 118}$ = 10.82, P = 0.001**
Current ALAN treatment x Evolved ALAN treatment			1	$F_{1, 118}$ = 0.55, P = 0.46

Table 4-3. Statistical models exploring the potential for local adaptation to ALAN (interaction between current treatment at F_{17} or F_{27} and evolved treatment) in life history traits of longevity and size in *D. melanogaster*. Besides the main variable of current and evolved ALAN treatment and their interaction, only those variables contributing to the minimal adequate model are reported. All averages are reported from untransformed data as the mean ± standard error, proportions or median (interquartile range).

Model Parameters	**Mean ± SE, Proportions or Median (interquartile range)**		**df**	**Statistic, P value**
ALAN Treatment	**0 lx**	**10 lx**		
Longevity (F_{17})				
a) Adult longevity (days)				
Females				
Current ALAN treatment	39 (35-43)	38 (32-40)	1	**χ^2= 4.98, P = 0.03**
Evolved ALAN treatment	39 (33-43)	37 (34-40)	1	**χ^2= 4.79, P = 0.03**
Current ALAN treatment x Evolved ALAN treatment			1	χ^2= 0.28, P = 0.60
Males				
Current ALAN treatment	39 (34-41)	39 (34-42)	1	χ^2= 0.51, P = 0.47
Evolved ALAN treatment	39 (34-42)	39 (34-41)	1	χ^2= 0.90, P = 0.34
Current ALAN treatment x Evolved ALAN treatment			1	χ^2= 0.04, P = 0.84
Size (F_{27})				
b) Wing length (mm)				
Current ALAN treatment	1.31 ± 0.01	1.28 ± 0.01	1	**$F_{1, 255}$ = 15.95, P < 0.001**
Evolved ALAN treatment	1.29 ± 0.01	1.30 ± 0.01	1	**$F_{1, 255}$ = 8.11, P = 0.01**
Current ALAN treatment x Evolved ALAN treatment			1	$F_{1, 255}$ = 2.01, P = 0.16
Sex			1	**$F_{1, 255}$ = 1489, P < 0.001**

Table 4-4. Statistical models exploring the potential for local adaptation in oxidative DNA damage (interaction between current treatment at F_{27} and evolved treatment) in *D. melanogaster.* Besides the main variable of current and evolved ALAN treatment and their interaction, only those variables contributing to the minimal adequate model are reported. Averages are the mean ± standard error.

Model Parameters	**Mean ± SE**		**Statistic, P value**
ALAN Treatment	**0 lx**	**10 lx**	
***Oxidative Damage (8-OhdG)* (µg)**			
Females			
Current ALAN treatment	0.026 ± 0.002	0.019 ± 0.002	**$F_{1, 48}$ = 8.45, P = 0.01**
Evolved ALAN treatment	0.023 ± 0.002	0.23 ± 0.002	$F_{1, 48}$ = 0.01, P = 0.93
Current ALAN treatment x Evolved ALAN treatment			$F_{1, 48}$ = 0.37, P = 0.55
Males			
Current ALAN treatment	0.022 ± 0.001	0.025 ± 0.002	$F_{1, 36}$ = 0.73, P = 0.40
Evolved ALAN treatment	0.025 ± 0.002	0.023 ± 0.002	$F_{1, 36}$ = 0.55, P = 0.46
Current ALAN treatment x Evolved ALAN treatment			$F_{1, 36}$ = 0.11, P = 0.74

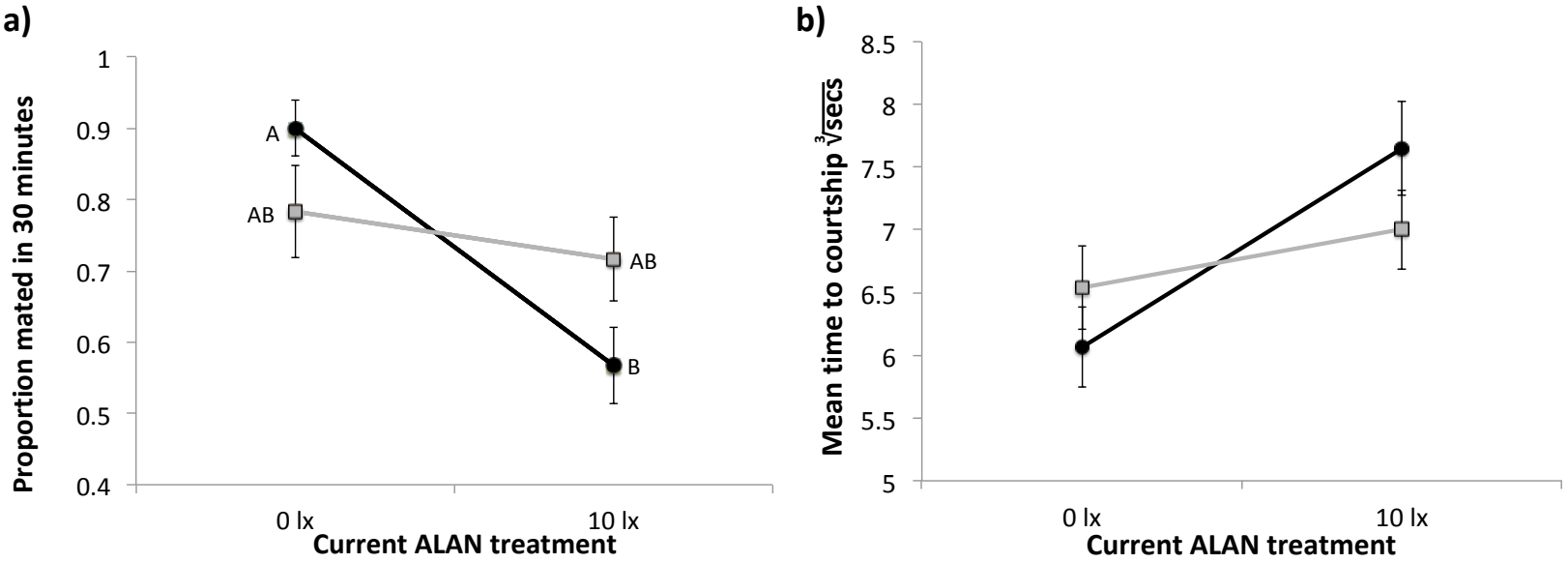


Figure 4-1. Graphs exploring differences in copulation commencement and time to courtship in F_{17} generation flies varying with current and evolved ALAN treatment, with local adaptation found in probability of mating. Circles and black lines denote 0 lx evolved ALAN treatment, squares and grey lines show 10 lx evolved ALAN treatment. **a)** Proportion of pairs successfully commenced mating within 30 minutes (P = 0.001 for current ALAN treatment; P = 0.22 for evolved ALAN treatment; P = 0.04 for current x evolved ALAN treatments); and **b)** Time to courtship (P = 0.22 for current x evolved ALAN treatments). Main effects between ALAN treatments not denoted on graphs. Uppercase letters denote significant interactions between current and evolved treatment groups. Error bars represent SE. For samples sizes see Supplementary Table 1 at the end of the Chapter 4.

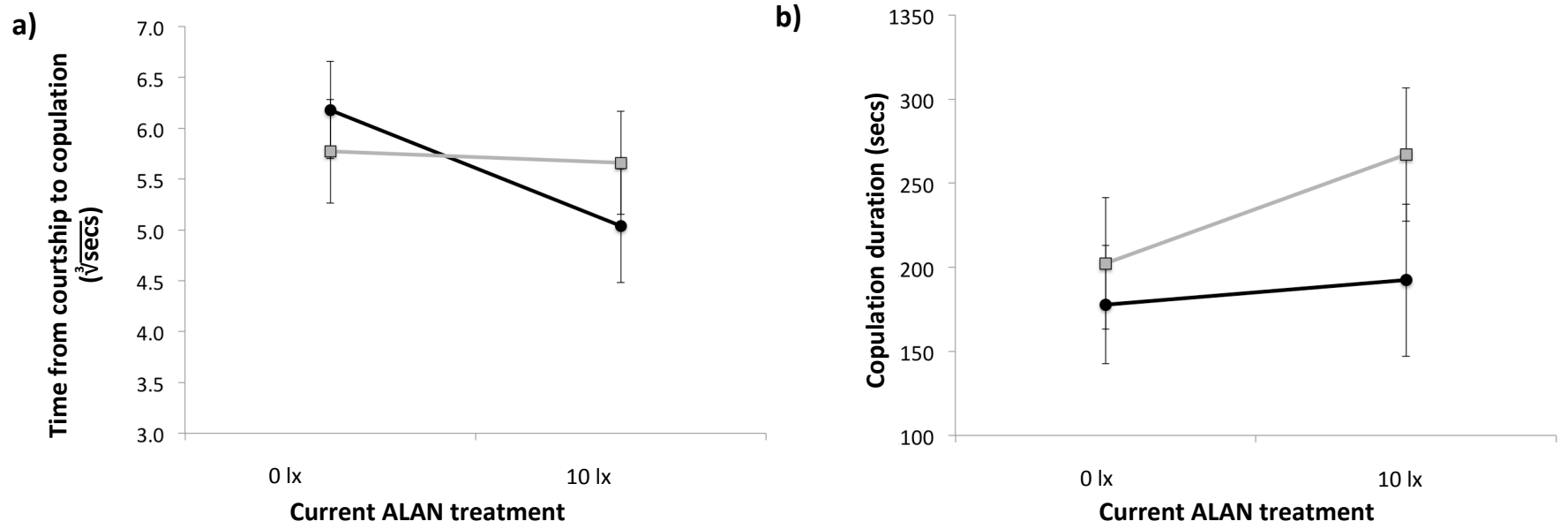


Figure 4-2. Graphs exploring differences in time from courtship to copulation and copulation duration in F_{17} generation flies varying with current and evolved ALAN treatment. No evidence of local adaptation was found. Circles and black lines denote 0 lx evolved ALAN treatment, squares and grey lines show 10 lx evolved ALAN treatment. **a)** Time from commencement of courtship to copulation (P = 0.48 for current x evolved ALAN treatments); and **b)** copulation duration (P = 0.66 for current x evolved ALAN treatments). Main effects between ALAN treatments not denoted on graphs. Error bars represent SE. For samples sizes see Supplementary Table 1 at the end of the Chapter 4.

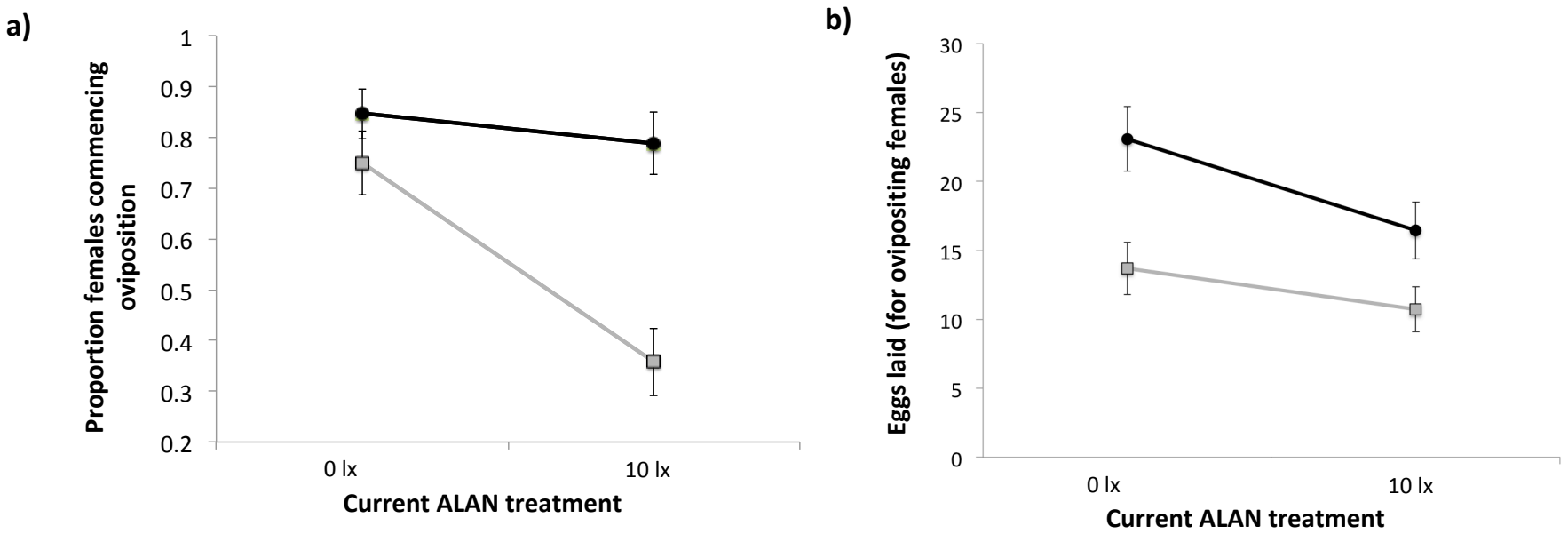


Figure 4-3. Graphs exploring differences in oviposition (F_{17}) in flies varying with current and evolved ALAN treatment. No evidence of local adaptation was found. Circles and black lines denote 0 lx evolved ALAN treatment, squares and grey lines show 10 lx evolved ALAN treatment. **a)** Proportion of females laying at least one egg (P = 0.08 for current x evolved ALAN treatments); and **b)** Number of eggs laid if oviposition commenced (P = 0.46 for current x evolved ALAN treatments). Main effects between ALAN treatments not denoted on graphs. Error bars represent SE. For samples sizes see Supplementary Table 1 at the end of the Chapter 4.

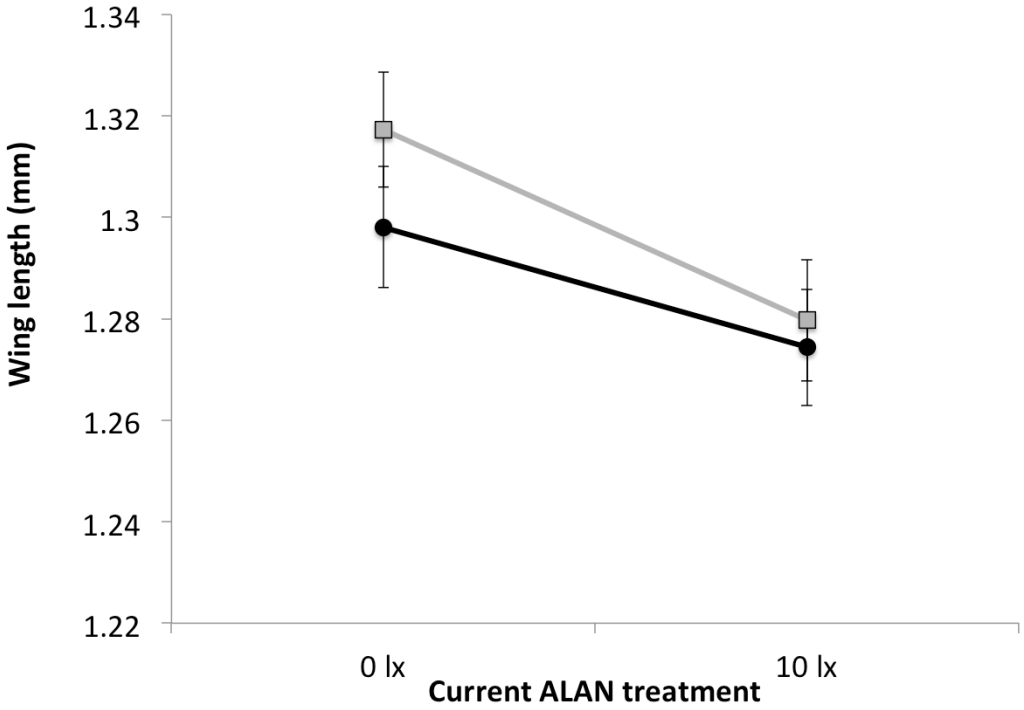


Figure 4-4. Graph exploring adult wing length (F_{27}) in flies varying with current and evolved ALAN treatment (P = 0.16 for current x evolved ALAN treatments). No evidence of local adaptation was found. Circles and black lines denote 0 lx evolved ALAN treatment, squares and grey lines show 10 lx evolved ALAN treatment. Main effects between ALAN treatments not denoted. Error bars represent SE. For samples sizes see Supplementary Table 1 at the end of the Chapter 4.

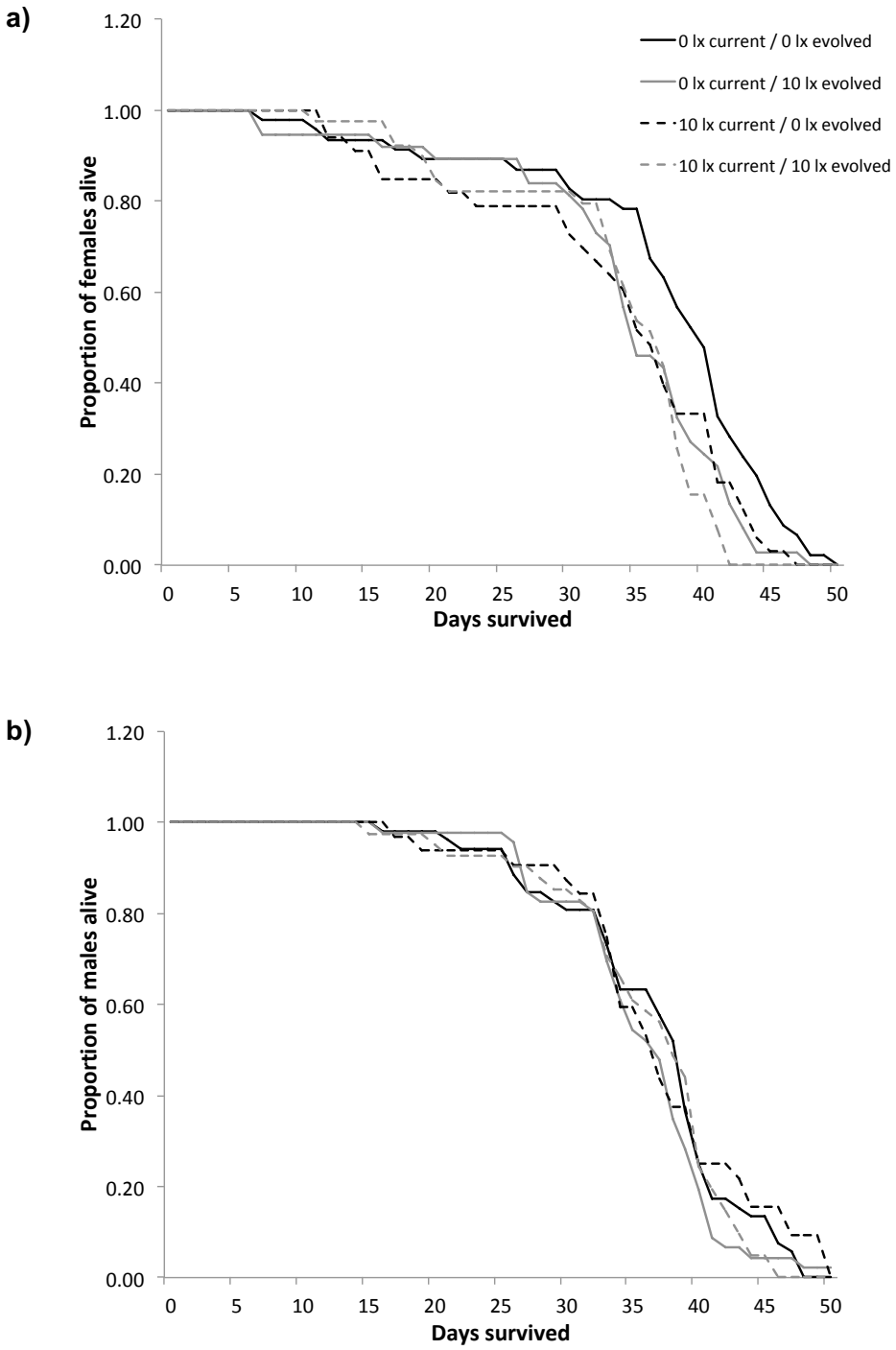


Figure 4-5. Survival curves varying with current and evolved ALAN treatment. No evidence of local adaptation was found in either sex. **a)** Female survival curve (P = 0.60 for current x evolved treatments); and **b)** Male survival curve (P = 0.84 for current x evolved treatments). Main effects between ALAN treatments not denoted on graphs. For samples sizes see Supplementary Table 1 at the end of the Chapter 4.

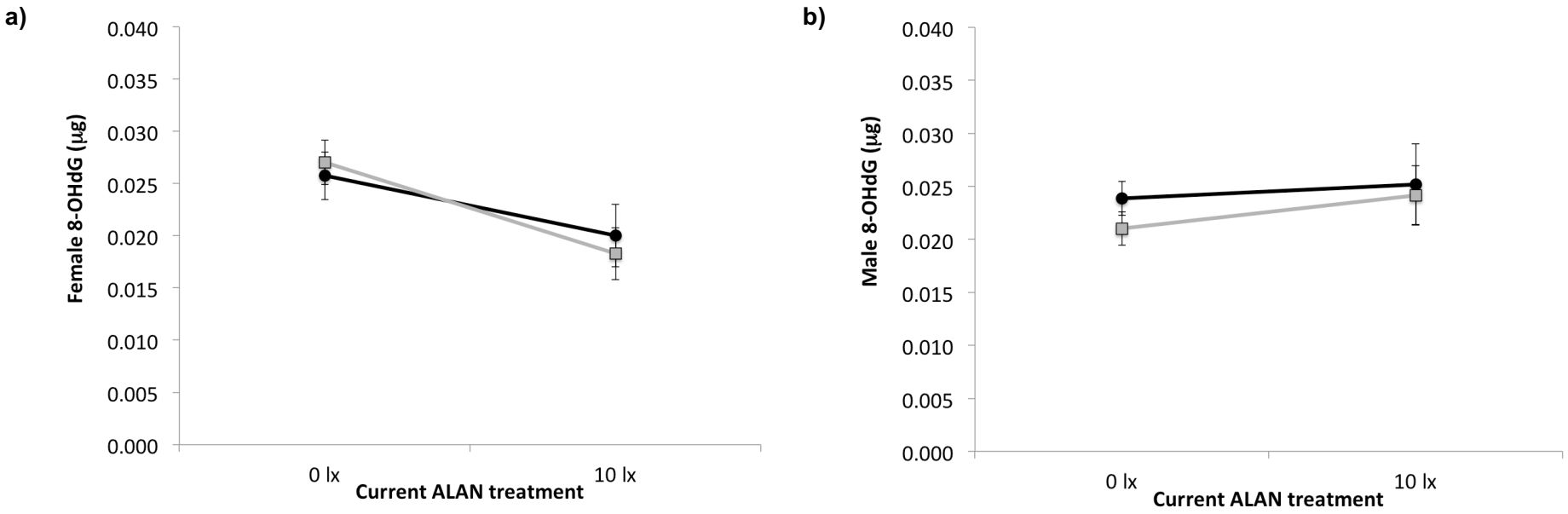


Figure 4-6. Graphs exploring differences in 8-OHdG (indicating DNA damage) at F_{27} generation varying with current and evolved ALAN treatment. No evidence of local adaptation was found. Circles and black lines denote 0 lx evolved ALAN treatment, squares and grey lines show 10 lx evolved ALAN treatment. **a)** 8-OHdG DNA damage in pooled samples of 30 females (P = 0.55 for current x evolved ALAN treatments); and **b)** 8-OHdG DNA damage in pooled samples of 30 males (P = 0.74 for current x evolved ALAN treatments). Main effects between ALAN treatments not denoted. Error bars represent SE. For samples sizes see Supplementary Table 1 at the end of the Chapter 4.

4.5 Discussion

Our experimental evolution paradigm using ALAN (in isolation to other potential urban stressors) allowed us to distinguish, for the first time, between plastic and evolved phenotypic responses associated with the presence of ALAN in our population. Our study confirmed the robustness of negative short-term (within two generations) phenotypic responses of exposure to light at night under laboratory conditions: individuals under 10 lx had disrupted mating behaviour, lower fecundity and reduced female longevity compared to flies under no light at night. Together these responses are likely to confer negative fitness under ALAN compared to dark nights and indicate the potential (as has been postulated, Swaddle et al., 2015, Hopkins et al., 2018) for ALAN to act as a driver for adaptive change. Our experimental evolution approach demonstrated that despite the relative consistency in the observed phenotypic responses following exposure to the presence of light at night, there was limited evidence that ALAN in a laboratory environment presented a sufficiently strong selection pressure to drive evolutionary change. Only mating propensity (the likelihood of mating within a 30 min assay period) responded to the imposed selection pressure under ALAN. In addition to short-term phenotypic effects on life history traits, we also found short-term physiological effects of ALAN for the first time, with a small reduction in adult size in both sexes when flies were exposed to 10 lx at night and, contrary to expectation, female flies (but not males) had lower levels of DNA damage (as measured by levels of 8-OHdG) compared to conspecifics under no light at night. The fitness consequences of these physiological effects of ALAN is unclear and they did not show adaptive change after evolution under ALAN. Overall, our study indicates that ALAN can act as a selection pressure to drive evolutionary change, but that its selective potential may be weaker than anticipated using this particular model system under benign conditions.

The short-term phenotypic responses to ALAN exposure in fitness traits, replicate our previous findings that ALAN at 10 lx generally disrupts mating behaviour (McLay et al., 2018, although different aspects of mating behaviour were affected), decreases fecundity and reduces longevity (McLay et al., 2017) in *D. melanogaster*, and confirm the potential for ALAN to drive adaptive change. However conversely, our experimental evolution results demonstrate the absence of broad-scale evolutionary change under light at night. This is perhaps surprising, particularly given direct selection evolution experiments in *D. melanogaster,* have

demonstrated that environmental stressors can induce local adaptation in fecundity (Rose et al., 1992, Wolfle et al., 2009), mating success (Zajitschek et al., 2016) and longevity (Rose et al., 1992). Given the robustness of the short-term negative phenotypic responses to ALAN, our experiment perhaps suggests that ALAN's selection pressure was weaker than predicted. Nonetheless, it was strong enough to promote variation in mating propensity: the significant interaction between current and evolved treatment indicating that the phenotypic response was not short-term and flies evolved under 10 lx ALAN were as equally likely to mate as flies evolved and maintained under dark nights (compared with flies evolved under 0 lx and then exposed to 10 lx). It is possible that our experimental design provided some additional direct selection pressure (in addition to ALAN), that resulted in selection of more rapidly mating flies (in both populations). In *D. melanogaster*, newly eclosed females are unreceptive to mating for approximately eight hours and then again after mating, for approximately 48 hrs (Manning, 1967). We allowed each generation of flies two to three days to mate before discarding adults, therefore those male flies that secured the first (and probably the only) mating with a female were the most likely to contribute to the next generation. We have demonstrated that approximately 80% of pairs under 0 lx mate within 30 minutes, so most males have the potential to contribute to the next generation under 0 lx. However, if some males under 10 lx are unable to swiftly secure mating, others (perhaps less affected by ALAN) may secure several copulations. Therefore, our procedures may have limited the number of males that successfully became sires under 10 lx. Further testing of this hypothesis would be possible by directly selecting for rapidly mating flies at each generation for the duration of the experimental evolution.

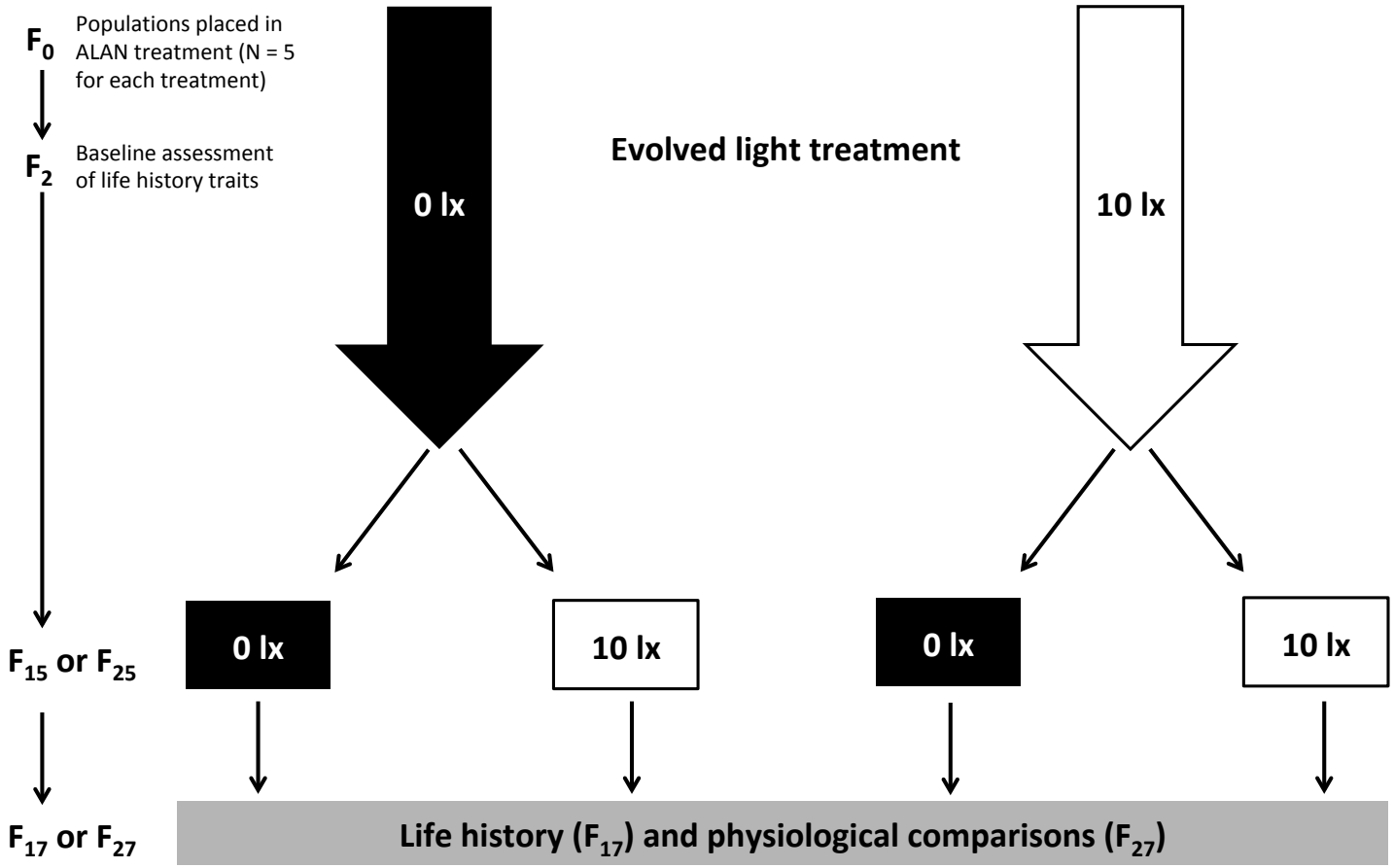
There are several possibilities why the other life history traits did not exhibit adaptation in our experiment, although reasonably, we would only expect the life history traits of mating and fecundity to demonstrate local adaptation in this model, as we did not allow our evolving populations to age beyond four days and the fitness consequences of smaller size and lower DNA damage under ALAN is untested. It is possible that crossing independently evolving populations (of the size used in our experiment) in the generation before assessing fitness traits, increased reproductive success through hybrid vigour (Margan et al., 1998) and masked any effects of evolution under 10 lx on fecundity. Additionally, standing genetic diversity within each independently evolving population is likely to differ at particular loci, resulting in

the potential for heterozygote crosses between each population to conceal adaptations in individual populations. Finally, in the comparatively benign laboratory environment, ALAN may not provide a strong enough pressure to generate selection in fecundity within the number of generations afforded in our model, or that the genetic variability of our founder population in relation to fecundity was insufficient to generate genetic change. Further experimental evolution research with additional stressors (for example, temperature or dietary restriction) and for more generations would verify whether this is the case.

In addition to confirming ALAN's effects on fitness traits, we add a previously untested but highly pertinent physiological trait that may be affected in part due to the presumed impact of ALAN on oxidative damage, namely DNA damage. Theoretically, exposure to light at night is anticipated to increase oxidative stress and induce oxidative damage, such as DNA damage, due to ALAN-induced reductions in the antioxidant melatonin (Reiter, 2002). However, the only studies to date undertaken under ecologically relevant ALAN intensity, show no correlation between dim ALAN and oxidative damage markers, in blood samples of either the great tit (*Pavus major*) (Raap et al., 2016, Casasole et al., 2017) or the wallaby *Macropus eugenii* (Dimovski and Robert, 2018) suggesting that the mechanism may be more complex than current presumed. Our data confirm this complexity, as here, we found females exposed to 10 lx ALAN had reduced DNA damage compared to their 0 lx conspecifics, irrespective of their evolved environment. This does not support the potential of dim ALAN to act as a mutagen to drive evolutionary change, however these results complement our previous experiment that found reduced levels of ROS levels (in ovaries) for 10 lx compared to 0 lx females (McLay et al., 2018). The reasons behind lower ROS and oxidative damage levels are unclear, but we suggest that it might arise because the imposed light treatments are linked to variation in female reproductive output. Although somewhat controversial, it is proposed that increased oxidative stress is a potential cost of reproduction (Alonso-Alvarez et al., 2017, Costantini, 2019). Female flies under 10 lx in our experiment invested less in reproduction (oviposition commencement and number of eggs laid), than flies under 0 lx, which may therefore have translated to lower levels of oxidative damage. A plausible alternative is that ALAN increases oxidative stress, thereby inducing an up-regulation of antioxidants sufficient to limit oxidative damage (Monaghan et al., 2009). A scenario not triggered under 0 lx conditions, where the levels of antioxidants may be more moderate and below thresholds that result in an

increased oxidative damage response. This has been suggested to explain increases in antioxidants produced in *Parus major* nestlings correlated with levels of oxidative damage in urban compared to rural environments (Salmon et al., 2018, although ALAN was not measured in this study). However, this potential increased investment in antioxidants is likely to be costly and entail trade-offs with investment in other life history traits (Monaghan et al., 2009). Indeed, we observed phenotypic responses of lower fecundity and smaller size (which are likely to be correlated (Lefranc and Bundgaard, 2000) but not tested together here) and shorter longevity but note that, as we only sampled oxidative damage at one time point (20 days adult age), it is unclear if only those flies with lower oxidative damage survived to this age, or if the observed comparative oxidative damage levels between 10 and 0 lx would persist until natural death. This is interesting, as the inverse relationship between longevity and oxidative damage (Barja, 2002) would suggest that flies under ALAN with lower oxidative damage would live longer, however this was not evidenced here. Therefore, further research into age specific changes in oxidative damage and its relationship to fecundity under ecologically relevant ALAN intensities and the implications for individual fitness is warranted, as our novel finding is of great interest.

In conclusion, we have demonstrated for the first time, that ALAN exposure (regardless of evolved ALAN treatment) results in smaller individuals and, intriguingly, lower levels of oxidative DNA damage in female *D. melanogaster*. The reasons behind these effects is uncertain and requires further research. Importantly, we have established that ALAN (in the absence of other urban stressors) induced limited local adaptation in mating propensity, but not other life history traits, in only 15 generations under laboratory conditions. It is likely its strength as a selective agent in combination with other urban stressors may be more robust and therefore, that ALAN can act as a driver of evolutionary change in urban environments.



Supplementary Figure 4-1. Experimental design for experimental evolution life history and physiological comparisons.

Supplementary Table 4-1. Samples sizes for all assays.

ALAN Treatment		**Sample sizes**										
		Baseline assessment of life history (F_2)				**Assessment of selection (F_{17} and F_{27})**						
						Life History					**DNA damage (F_{27})** (Pooled samples of 30 flies)	
Current treatment	***Evolved treatment***	***Mating***	***Fecundity***	***Longevity***		***Mating (F_{17})***	***Fecundity (F_{17})***	***Longevity (F_{17})***		***Wing size (F_{27})***	***Female 8-OHdG (F_{27})***	***Male 8-OHdG (F_{27})***
				Female	***Male***			***Female***	***Male***			
0 lx	0 lx	58	45	46	46	60	52	46	52	62	12	9
0 lx	10 lx					60	44	38	46	64	12	9
10 lx	0 lx	60	36	37	37	60	33	33	32	64	12	9
10 lx	10 lx					60	42	39	41	64	12	9

Original paper

Chapter 5:

Effects of dietary melatonin supplementation and light at night on life history traits in *Drosophila melanogaster*

by

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This work is currently being prepared for publication

5.1 Abstract

The link between exposure to artificial light at night (ALAN) and taxa-wide shifts in animal behaviour and physiology, including key life history traits such as longevity, fecundity, growth and development are increasingly demonstrated. A proposed mechanism underpinning these observed effects is the direct impact of light on the photosensitive melatonin pathway. Melatonin is typically nocturnally endogenously produced and is involved in regulating circadian rhythms as well as being a powerful antioxidant. Correlational studies confirm links between exposure to dim ALAN and reductions in nocturnal melatonin concentrations. Moreover, melatonin is known to be involved in processes regulating aspects of key life history traits. However, a direct causal link between shifts in life history traits and variation in melatonin in the presence of ALAN, is currently lacking. Here, we investigated simultaneously, the effect of lifetime dietary supplementation of melatonin (0 μg, 50 μg or 100 μg per ml of nutrient medium) and the presence of ALAN (0 lx or 10 lx) on the life history traits of longevity, fecundity and development in *Drosophila melanogaster*. These doses were previously demonstrated to be biologically relevant in this species. Our results confirm the negative impact of ALAN on development (altered eclosion patterns and reduced size), longevity and fecundity in *D. melanogaster.* However, despite replicating doses in a previous study, melatonin supplementation was found to be detrimental, resulting in negative fitness consequences (reduced longevity for flies under dark nights and disrupted eclosion patterns regardless of ALAN treatment). This suggests that the dosing regimen used was potentially toxic when administered chronically over a lifetime. Our data provide a potential cautionary note, that given the known roles of melatonin as a driver of circadian rhythm and antioxidant, a different acute or intermittent administration regimen should be explored in future studies, to disentangle whether the dose and/or the chronic administration was responsible for the observed effects. Identifying this is therefore pivotal in optimising an experimental design to explore the role of melatonin as a potential mechanism driving phenotypic change under ALAN.

Introduction

The global presence of artificial light at night (ALAN) is increasing annually in both distribution and intensity resulting in the suppression and potential masking of natural light cycles (Gaston et al., 2014, Kyba et al., 2017). ALAN is associated with a range of physiological and behavioural changes in individual organisms, species and communities (for reviews see Gaston et al., 2014, Tierney et al., 2017a, Dominoni and Nelson, 2018, Russart and Nelson, 2018). At the level of the individual, ALAN is linked to shifts in several key life history traits in animals, including reduced longevity (McLay et al., 2017, Willmott et al., 2018), lower fecundity (McLay et al., 2017, Willmott et al., 2018), changes in growth and size (with ALAN correlated with larger individuals in some species (Aubrecht et al., 2015, Durrant et al., 2018) and smaller size in others (van Geffen et al., 2014, Raap et al., 2016, Willmott et al., 2018, McLay et al., Chapter 4) and disruptions to development rates and timing of maturation (Thakurdas et al., 2009, Dominoni et al., 2013c, van Geffen et al., 2014, Robert et al., 2015, Durrant et al., 2018, Willmott et al., 2018). Understanding the mechanism underpinning these observed changes is essential and yet is largely unexplored in this context.

A potential mechanism explaining the physiological and behavioural changes associated with ALAN, is that its presence suppresses the endogenous photosensitive production of the indolamine, melatonin (N-acetyl-5-methoxytryptamine). Melatonin has been identified in all major taxa of organisms (Tan et al., 2010), including invertebrates (Vivien-Roels and Pevet, 1993) and, in animals, its synthesis is typically highest at night (Tan et al., 2010). Thus, one of melatonin's primary functions is as a signal of darkness (Arendt and Skene, 2005), operating as a direct messenger between the biological master clock in the brain (in insects) which is entrained by light (Dubowy and Sehgal, 2017) and peripheral tissues, where it is involved in modulation of a diverse range of physiological functions (for reviews, see Pandi-Perumal et al., 2006, Tan et al., 2010). Consequently, melatonin directs many circadian and seasonal rhythms and affects associated aspects of animal behaviour and physiology (Zawilska, 1996, Tan et al., 2010), although the master clock is also able to influence organs and tissues via direct neuronal control (Buijs et al., 2003). Additional to its circadian function, melatonin is a powerful antioxidant (Tan et al., 2010), involved in mitigating oxidative stress in macromolecules, cells, tissues, organs and organisms (Hardeland, 2005, Anisimov et al., 2006).

Several experimental studies have established that ALAN correlates with lower nocturnal melatonin levels in vertebrate species, such as in the Syrian hamster *Mesocricetus auratus* (Brainard et al., 1982), fish *Rutilis rutilis* and *Perca fluviatilis* (Bruning et al., 2015, Bruning et al., 2018), blackbird *Turdus merula* (Dominoni et al., 2013a), great tit *Parus major* (de Jong et al., 2016) and the tammar wallaby *Macropus eugenii* (Robert et al., 2015, Dimovski and Robert, 2018). However, these studies do not provide evidence of a causal link between reduced melatonin levels and the life history shifts observed under ALAN. Moreover, as melatonin concentrations are typically extremely low (Vivien-Roels and Pevet, 1993), vary throughout a 24hr cycle (Vivien-Roels and Pevet, 1993) and the timing of the daily melatonin peak is not consistent across individuals (Vivien-Roels and Pevet, 1993), estimating the causal relationship between reduced levels of melatonin in individuals using a sampling experiment and observed variation in life history traits is logistically challenging.

One potential method to address the above challenges, is to provide melatonin exogenously and assess the biological response. In both vertebrates and invertebrates, melatonin supplementation (although not specifically with ALAN as an experimental paradigm), is linked to improved survival (Coto-Montes and Hardeland, 1999, Bonilla et al., 2002, Bonilla et al., 2006, Tan et al., 2010, Teran et al., 2012, Medina-Leendertz et al., 2014), buffering of the immune system (Carrillo-Vico et al., 2013, Jones et al., 2015), increases in early developmental growth (Brockus et al., 2016) and slower adult maturation (Pandi-Perumal et al., 2006, Niva and Takeda, 2003). Vertebrate focussed studies also demonstrate melatonin supplementation increases reproductive fitness (Delgadillo et al., 2001, Bertrand et al., 2006, Casao et al., 2010, Cruz et al., 2014, Talpur et al., 2018). Conversely, melatonin's role in reproduction in invertebrates is largely untested, however it appears to influence ovarian maturation (Sainath and Reddy, 2011), mating speed and oviposition rate (Finocchiaro et al., 1988). Despite melatonin's influence on life history traits and the relationship between ALAN and levels of circulating nocturnal melatonin, no study to date has explored the relationships between melatonin supplementation, the presence of ecologically relevant intensities of light at night and life history traits.

Here, we investigated simultaneously, the biological effect of variation in lifetime supplementation with dietary melatonin (0 μg/ml, 50 μg/ml or 100 μg/ml – see below) and exposure to dim ALAN (10 lx, comparable to street light or 0 lx, dark night) in a model

invertebrate, *Drosophila melanogaster*. This is an ideal species for this study as it displays reduced longevity and fecundity under ALAN (McLay et al., 2017, McLay et al., 2018). Based on previous studies, we predicted that flies reared under ALAN would have reduced longevity, fecundity and altered growth and development patterns. Given melatonin's likely role in circadian rhythm and as an antioxidant, we further predicted that supplementation with dietary melatonin should modulate the negative effects of ALAN.

5.2 Methods

5.2.1 Fly Stocks

Experimental flies were sourced from a stock population of *Drosophila melanogaster* (100 adult females and 50 adult males) collected in April 2015 from Oak Ridge Winery (Victoria, Australia; co-ordinates: -37.686908, 145.457438) and then maintained in the laboratory for 70 generations at 28 ± 1°C under standard conditions (see McLay et al. (2017). Stock flies were maintained at a density of approximately 100 flies per generation (1:1 sex ratio).

5.2.2 ALAN Treatments

Experimental flies were maintained under identical daytime light levels (12 hours at 2600 lx - equivalent to an overcast day, 6800 kelvin); but were exposed to one of two night-time light levels: 12 hr at 0 lx (0 kelvin); or 10 lx (5900 kelvin) (for further details, see McLay et al., 2017). To ensure no incubator bias, we rotated flies and their corresponding ALAN treatment between four incubators every two to three days and randomly repositioned fly vials within each incubator at each move.

5.2.3 Melatonin Treatments

Within each ALAN treatment, we allocated experimental flies to one of three dietary supplementation treatments of melatonin in nutrient media: 0 μg/ml melatonin (no melatonin control), 50 μg/ml melatonin or 100 μg/ml melatonin. We chose 100 μg/ml melatonin as the maximum concentration, as several previous studies using *Drosophila* demonstrated its positive impact on life span when provided as a constant supplement in nutrient media for various life stages: adult only (Bonilla et al., 2002); juvenile to 5-day old adults (Reynolds, 2018); and throughout juvenile and adult life-stages (Teran et al., 2012). The intermediate

concentration of 50 μg/ml melatonin allowed us to assess whether there was any dose dependent response in any of our assays. To create the supplemented media, powdered melatonin (Glentham Life Sciences, Corsham, UK) was solubilised in ethanol and diluted to working concentrations of 10 and 5 mg/ml respectively, before adding it to cooled nutrient media (below 45°C) during preparation. All treatments (including the no melatonin control) had a final concentration of ethanol in the nutrient medium of 0.2%. The nutrient media were prepared weekly from aliquots of the same original stock solutions, and either transferred to vials (7 ml per vial) or left as a flat sheet (10 mm thick) and then stored in the dark at 4°C.

5.2.4 Experimental Flies

To ensure that eggs contributing to the experimental flies were produced under varying melatonin concentrations, we reared flies under ALAN treatment for one generation and then for a further generation under both their allocated ALAN and melatonin treatment. We placed five F_0 stock bottles (250ml) containing approximately 50 recently emerged female and male flies under either 0 lx or 10 lx ALAN treatments and allowed them to mate and oviposit for one day. We then discarded adult flies and allowed the F_1 offspring to mature to adult, after which we permitted them to mature and mate for three days within their eclosion bottles before placing them in melatonin treatment. For each melatonin/ALAN treatment combination, we collected two three-day old F_1 females from each of the five replicate ALAN treatment bottles and placed them together in vials (10 females per vial (96 mm x 25 mm), five vials per melatonin/ALAN treatment combination). We allowed them to oviposit for 18 hours, after which we discarded the females and reared the F_2 offspring to adult. To ensure that developing larvae were continually exposed to melatonin, we added a disk (20 mm x 7 mm) of the allocated melatonin medium every two to three days to developing F_2 vials. The disk was gently pushed into the top of the medium until it formed a flat surface. Preliminary trials adding identical sized disks of standard medium (0 μg/ml melatonin) to vials, resulted in comparable egg to adult survival to non-manipulated vials (proportion of eggs surviving to adult eclosion with a nutrient disk = 0.84 ± 0.10; without a nutrient disk = 0.71 ± 0.19; $F_{1, 21}$ = 0.25, P = 0.62). On the first day of F_2 adult eclosion, we transferred eclosed flies to new melatonin/ALAN treatment vials (maintaining parental vial groupings) and allowed adults to mate for 24 hours before assaying fitness traits of fecundity and longevity (following McLay et

al., 2017, McLay et al., 2018). We conducted the experiment in two blocks. All assays were conducted on F_2 or F_3 generation flies as indicated below.

5.2.5 Longevity and female fecundity (F_2)

We assessed female longevity at the population (higher density and likely a more stressful environment) and at the individual level.

Population level longevity – To assess longevity in a competitive group environment, we transferred 84 same-sex groups of 20, mated flies to nutrient vials containing their allocated melatonin treatments and maintained them under their ALAN treatment until death (N = 84 vials consisting of 1680 flies). Flies were transferred to new treatment vials every two to three days and checked daily for deaths.

Individual female longevity – To assess individual female longevity and fecundity, we transferred 152 mated male and female pairs to individual vials maintaining their allocated melatonin and ALAN treatments. Males were discarded after five days and females were transferred to a new vial every two to three days (except for day six and 12, see below) until their death.

Female fecundity – To explore variation in offspring production, on days six and 12 of adult life (*sensu* McLay et al., 2017, McLay et al., 2018), surviving adult females (N = 266) were each transferred to an individual vial and allowed to oviposit for 18 hours (at which time eggs begin to hatch at 28^{o}C), after which time the female was discarded. The resulting eggs were allowed to develop to adulthood under the same treatment conditions as their parents and eclosed F_3 flies counted daily for a week following the first day of emergence, after which time vials were discarded.

5.2.6 Offspring development time and size (F_3)

Finally, to assess differences in offspring development, we recorded the total number of F_3 offspring that emerged each day for a week after eclosion commenced. Emerging flies from day one of eclosion were transferred to ethanol and, for a sub-sample (N = 449), the left wing was subsequently measured as a proxy for adult size (McLay et al., 2017). Wing size was taken as the intersection of the anterior cross vein and the longitudinal vein 3 to the intersection of

longitudinal vein 3 with the distal wing margin as per McLay et al. (2017). Wings were photographed under a compound microscope (Olympus BX50) with attached camera (Olympus U-TV0.63XC) and measurements made using Image J software (NIH, Rockville, USA). All wing measurements were undertaken blind to treatment.

5.2.7 Statistical Analyses

We performed all analyses in R (R Core Team, 2016). Before analysis, data were assessed for normality and transformed where appropriate. We used Cox's proportional hazard regression model to assess longevity. For F_2 cohort longevity we used the package *coxme* (Therneau, 2012) and for F_2 individual female longevity, *coxph* (Therneau, 2012). For all other analyses, we used the package *lme4* (Bates et al., 2015). F_2 Female fecundity analysis was bimodal, therefore we analysed the number of vials that produced any offspring separately to the number of offspring produced in vials that produced any offspring. The number of vials that contained any offspring was analysed using a generalised linear mixed model (GLMER), with a binomial error distribution and logit link function. We used a linear mixed effects model, with REML (restricted maximum likelihood) for the total number of F_3 offspring produced in vials that had any offspring and for the proportion of total F_3 offspring that emerged on the first day of eclosion (almost all flies eclosed in the first two days of eclosion, so this metric was used for analysis). F_3 wing size was analysed using a linear model. For all initial analyses we used ALAN treatment, melatonin treatment, block and their interaction as fixed categorical variables. In all cases, there was no interaction between block, ALAN treatment and melatonin treatment and therefore, for final analysis block was included as a random variable. In F_2 longevity analysis, we included sex as a fixed categorical variable, vial cohort as a random effect in vials with multiple flies and total eggs laid as a continuous variable for individually housed females. For F_2 female fecundity analysis and proportion of flies to emerge on the first day of eclosion, we included female age as a fixed categorical variable (and interactions) and female identification as a random effect. The total number of F_3 offspring to eclose from a vial was included in the analysis of proportion of flies to eclose on the first day of eclosion. Sex was included as a categorical variable in F_3 wing size analysis. The significance of parameters was assessed using hierarchical backwards stepwise deletion, dropping terms from the model (except the main parameters of interest, ALAN and melatonin treatments) where $P > 0.01$. 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 exclusion. Post hoc Tukey's tests were used in the package *multcomp* (Hothorn et al., 2008) to determine differences between groups. The level of statistical significance was taken as $P < 0.05$.

5.3 Results

5.3.8 Longevity and female fecundity (F_2)

Population level longevity – There were significant main effects of melatonin concentration, ALAN treatment and sex ($P < 0.001$ for all, Table 5-1a), however these patterns were driven by significant interactions between treatments. There was significant variation in longevity across the six resulting ALAN and melatonin treatments (interaction between ALAN treatment and melatonin treatment: $P = 0.022$; Table 5-1a, Figure 5-1). Post hoc tests revealed that 0 lx flies in the control melatonin (0 μg/ml) treatment survived longer than all other treatments and 10 lx flies supplemented with 100 μg/ml of melatonin died earlier than all 0 lx flies (i.e. regardless of their melatonin treatment). Longevity across all other treatments was comparable. There was also an interaction between ALAN treatment and sex ($P < 0.001$, Table 5-1a). Post hoc tests revealed that females reared under 0 lx survived longer than all other treatments, but all other treatment combinations were comparable.

Individual female longevity – When reared individually, 10 lx females survived for less time than 0 lx flies ($P = 0.004$, Table 5-1b). However, longevity was comparable for all melatonin treatments ($P = 0.427$, Table 5-1b), with no interaction between ALAN and melatonin treatment.

Female fecundity – Flies maintained under 10 lx were less likely to produce offspring ($P = 0.007$; Table 5-1c) and produced fewer offspring than flies maintained under 0 lx ($P = 0.017$, Table 5-1d, Figure 5-2a). In contrast, the probability of producing any offspring ($P = 0.772$, Table 1c) or the number of offspring produced was unrelated to melatonin treatment ($P = 0.324$, Table 5-1d). The number of adults emerging from vials that successfully produced offspring was lower for 12 d compared to 6 d old females ($P = 0.002$, Table 5-1d).

5.3.9 Offspring development time and size (F_3)

The proportion of flies emerging on the first day of eclosion was lower for 10 lx flies compared to 0 lx flies ($P < 0.001$, Table 5-1e, Figure 5-2b) and for 6 d old compared to 12 d old females ($P < 0.001$, Table 5-1e). Additionally, a higher proportion flies emerged on the first day of eclosion in the presence of either 50 μg/ml or 100 μg/ml melatonin, compared to the no melatonin control (significant main effect of melatonin: $P = 0.002$, Table 5-1e, see Figure 5-2b for posthoc comparisons). Adult flies under 10 lx had smaller wings than the 0 lx flies ($P < 0.001$, Table 5-1f, Figure 5-2c) and females had bigger wings than males ($P < 0.001$, Table 5-1f). However, wing size was not related to melatonin treatment ($P = 0.242$, Table 5-1f).

Table 5-1. Statistical models exploring the effect of ALAN and melatonin treatment on life history traits in *D. melanogaster*. Beside the main variables of ALAN treatment, and melatonin treatment, only those variables contributing to the minimum adequate model are reported. Categorical variables and the order in which they are reported are given in parentheses. Statistics are reported as median and interquartile range, proportion or mean ± SE as appropriate. Different subscript letters denote significant differences between treatment groups.

Model Parameters	**Variable details**			**df**	**Statistic, P value**
Variable of interest and order in brackets	**Median (IQR: 25%-75%) / Proportion / Mean ± SE**				
Longevity and female fecundity (F_2)					
Adult longevity					
a) Population level longevity (days)					
ALAN treatment (0 lx, 10 lx)	32 (28-37)	29 (25-33)		1	**χ^2 = 24.79, P < 0.001**
Melatonin treatment (0μg/ml, 50μg/ml, 100μg/ml)	32 (27-37)$_A$	30 (26-35)$_B$	30 (25-35)$_B$	2	**χ^2 = 29.87, P < 0.001**
Sex (F, M)	32 (28-37)	29 (25-33)		1	**χ^2 = 60.80, P < 0.001**
ALAN treatment x melatonin treatment				2	**χ^2 = 7.64, P = 0.022**
ALAN treatment x sex				1	**χ^2 = 14.83, P < 0.001**
b) Individual female longevity (days)					
ALAN treatment (0 lx, 10 lx)	32 (23-38)	30 (18-35)		1	**χ^2 = 8.13, P = 0.004**
Melatonin treatment (0 μg/ml, 50 μg/ml, 100 μg/ml)	31 (20-38)	30 (23-35)	32 (16-38)	2	χ^2 = 1.70, P = 0.427
Female fecundity					
c) Number of vials that produced any offspring					
ALAN treatment (0 lx, 10 lx)	115/140 (82%)	81/126 (64%)		1	**χ^2 = 7.22, P = 0.007**
Melatonin treatment (0 μg/ml, 50 μg/ml, 100 μg/ml)	66/86 (77%)	70/98 (71%)	60/82 (73%)	2	χ^2 = 0.52, P = 0.772
Maternal age (D6, D12)	109/139 (87%)	87/127 (69%)		1	χ^2 = 4.18, P = 0.041
d) Number of offspring produced in vials that had any offspring (log transformed)					
ALAN treatment (0 lx, 10 lx)	16 (9-26)	12 (5-23)		1	**χ^2 = 5.72, P = 0.017**
Melatonin treatment (0 μg/ml, 50 μg/ml, 100 μg/ml)	16 (7-22)	14 (9-25)	15.5 (5-30)	2	χ^2 = 2.25, P = 0.324
Maternal age (D6, D12)	9 (18-28)	5 (12-21)		1	**χ^2 = 9.41, P = 0.002**
Offspring development time and size (F_3)					
e) Proportion of total offspring that emerged on day 1 of eclosion					
ALAN treatment (0 lx, 10 lx)	0.64 ± 0.03	0.48 ± 0.04		1	**χ^2 = 14.34, P < 0.001**
Melatonin treatment (0μg/ml, 50μg/ml, 100μg/ml)	0.47 ± 0.04$_A$	0.62 ± 0.03$_B$	0.64 ± 0.04$_B$	2	**χ^2 = 11.81, P = 0.002**
Maternal age (D6, D12)	0.49 ± 0.03	0.69 ± 0.03		1	**χ^2 = 31.13, P < 0.001**
f) F_3 wing length (mm)					
ALAN treatment (0 lx, 10 lx)	1.34 ± 0.01	1.32 ± 0.01		1	**$F_{1,449}$ = 20.62, P < 0.001**
Melatonin treatment (0 μg/ml, 50 μg/ml, 100 μg/ml)	1.33 ± 0.01	1.33 ± 0.01	1.32 ± 0.01	2	$F_{2,449}$ = 1.43, P = 0.242
Sex (F, M)	1.42 ± 0.003	1.24 ± 0.002		1	**$F_{1,449}$ = 2292.27, P < 0.001**

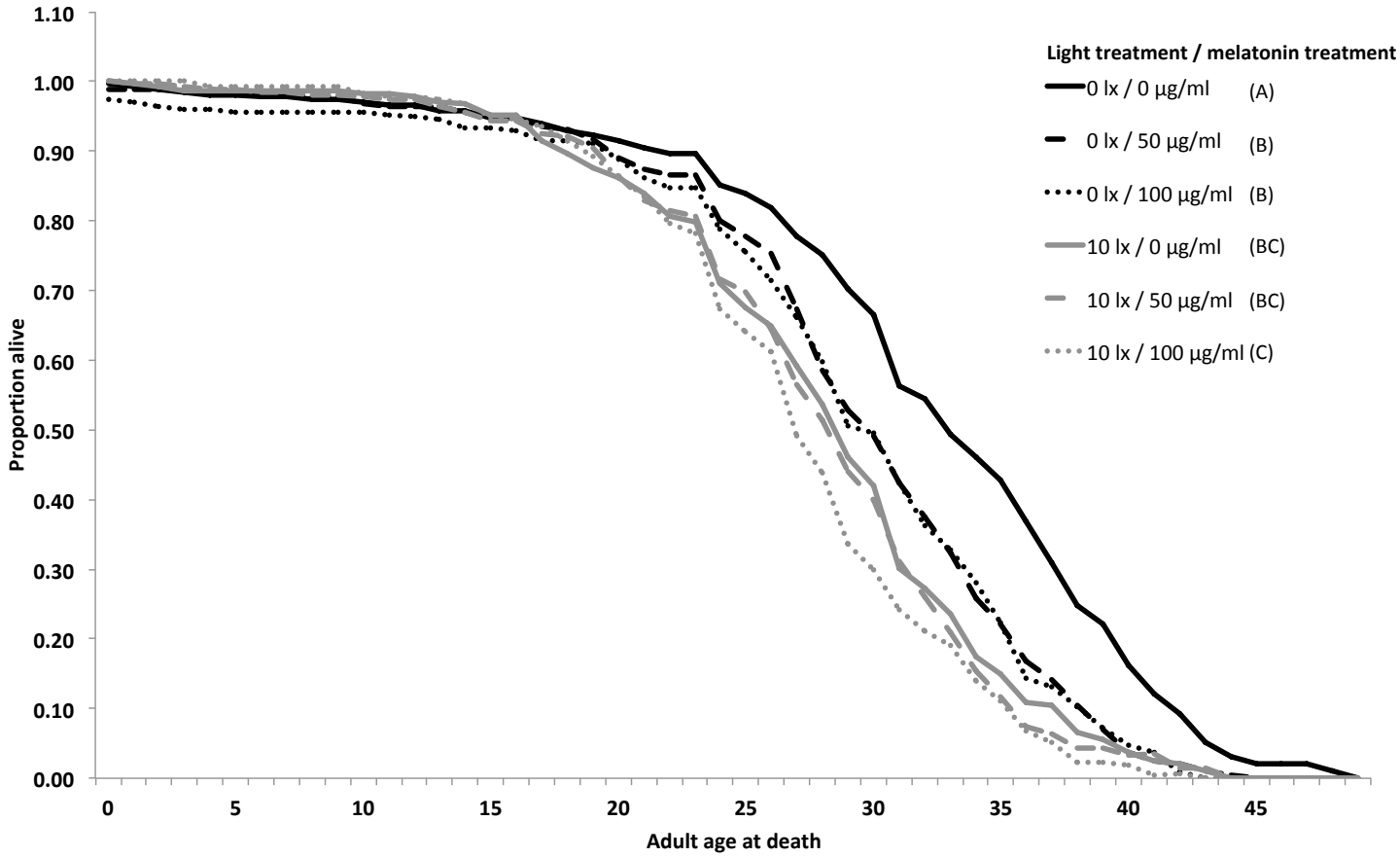


Figure 5-1. Adult survival curves from Cox's proportional hazard model for male and female *D. melanogaster* population vials under different ALAN/melatonin treatments (P = 0.022 for ALAN x melatonin treatment interaction, N = 14 vials for each treatment of: 269 flies for 0 lx/0 µg/ml; 274 flies for 0 lx/50 µg/ml; 269 flies for 0 lx/100 µg/ml; 275 flies for 10 lx/0 µg/ml; 271 flies for 10 lx/50 µg/ml; 269 flies for 10 lx/100 µg/ml). Upper case letters in figure legend denote differences between ALAN/melatonin treatments.

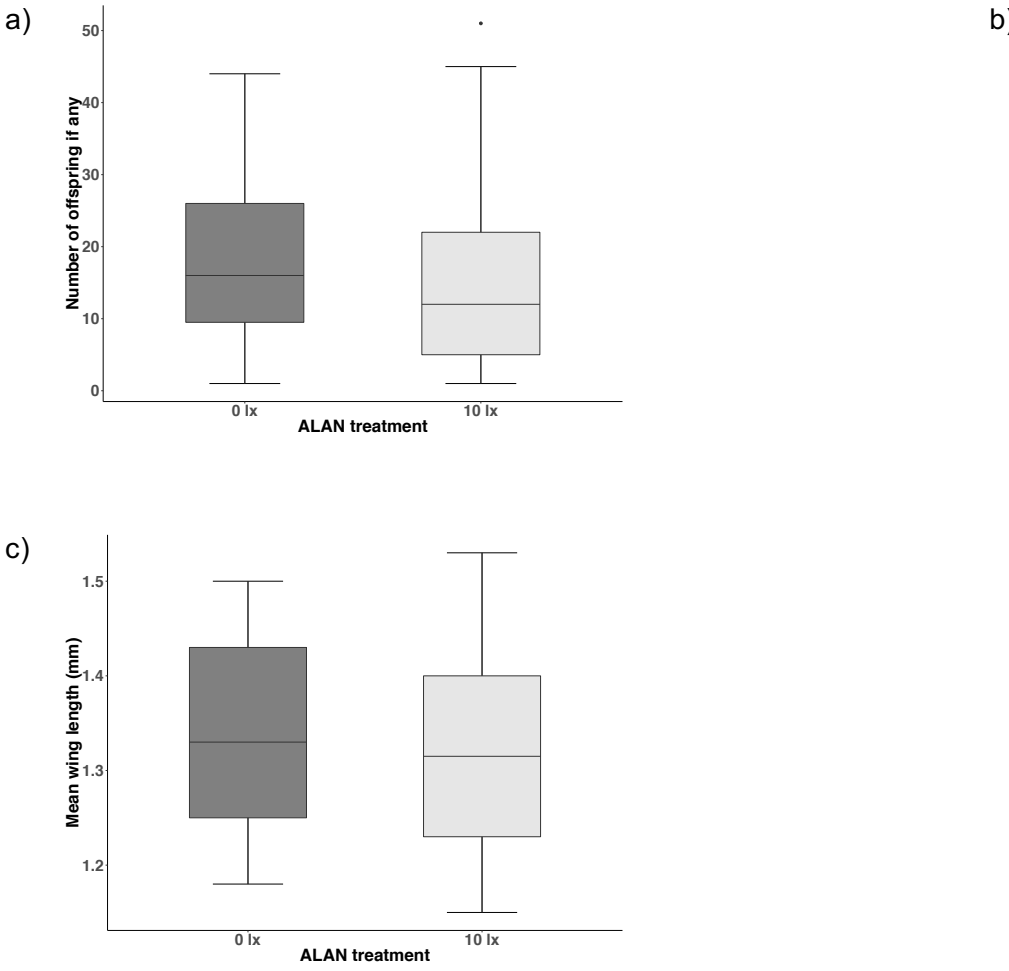


Figure 5-2. Graphs exploring differences in offspring production and fly size under varying ALAN and melatonin treatments **a)** Number of offspring produced in vials that produced any offspring for each ALAN treatment (P = 0.017: N = 115 for 0 lx; N = 81 for 10 lx). **b)** Proportion of flies to emerge on day one of eclosion for each vial in ALAN/ melatonin treatment (P < 0.001 for ALAN treatment, P = 0.002 for melatonin treatment: N = 39 for 0 lx/0 μg/ml; N = 36 for 0 lx/50 μg/ml; N = 40 for 0 lx/100 μg/ml; N = 27 for 10 lx/0 μg/ml; N = 34 for 10 lx/50 μg/ml; N = 20 for 10 lx/100 μg/ml). Dark grey bars represent 0 lx and light grey bars 10 lx ALAN treatments. Uppercase letters denote differences between melatonin treatments, lower case letters denote differences between ALAN treatments. **c)** Variation in F_3 wing length under different ALAN treatments (P < 0.001: N = 221 for 0 lx; N = 228 for 10 lx). All plots display median, quartiles and range of data.

5.4 Discussion

This study has three key findings. First, we confirm that *D. melanogaster* maintained under ALAN had lower fecundity and reduced longevity compared to flies maintained under 0 lx at night (McLay et al., 2017, McLay et al., 2018). Second, we demonstrate that ALAN disrupts eclosion patterns and reduces the size of early eclosing adults. Third, and contrary to our prediction, we show that lifetime supplementation of melatonin is potentially toxic. Despite being administered at concentrations and by methods previously demonstrated to have a positive biological effect in this species (Izmaylov and Obukhova, 1999, Bonilla et al., 2002, Teran et al., 2012, Medina-Leendertz et al., 2014), offspring exposed to melatonin eclosed sooner (regardless of ALAN treatment) and had reduced longevity when housed as a population under dark night (but not ALAN) conditions.

The negative effect of exposure to dim ALAN on longevity may have arisen through mutually non-exclusive mechanisms. ALAN may promote circadian shifts leading to altered rhythmic behaviours associated with rest/activity and oviposition patterns, which have been linked to decreased lifespan in *D. melanogaster* (Kumar et al., 2005). Alternatively, ALAN is related to the upregulation of systems dedicated to health maintenance, such as immune function (Moore and Siopes, 2000, Bedrosian et al., 2011b, Durrant et al., 2015) and potentially antioxidant capacity (McLay et al., 2018), which may lead to a trade-off with longevity (Monaghan et al., 2009). ALAN's effects on fecundity may also be the result of several potentially interconnected mechanisms that are likely due to indirect effects of ALAN, as the direct presence of light *per se* is unlikely to affect egg laying, given oviposition in *D. melanogaster* retains rhythmicity even under constant darkness or bright light (Howlader and Sharma, 2006). One explanation is that ALAN could affect fecundity though ALAN-induced changes to circadian rhythm and endogenous melatonin production which may indirectly affect reproductive physiology through hormonal disruption (for a review see Reiter, 1995), such as to the juvenile hormone (JH), necessary for the synthesis of yolk proteins and vitellogenesis in *D. melanogaster* (Howlader and Sharma, 2006). Notwithstanding, we note that overt changes to ovarian characteristics appear not to be correlated with ALAN as ovarian egg number and ovarian size appear comparable between ALAN treatments in this population (McLay et al., 2018). However, this is not evidence of egg quality or of whether these ovarian eggs will be laid. Moreover, we are unable to discount the possibility that ALAN-induced

alteration to antioxidant capacity causes more subtle changes to ovarian physiology: reactive oxygen species (ROS) levels in ovaries are lower under ALAN, potentially affecting cell signalling and/or egg release (McLay et al., 2018). Finally, potential interactions between circadian and antioxidant disruption under ALAN may affect fecundity by disrupting mating behaviour. Altered mating behaviour under ALAN is demonstrated in a range of invertebrates, including *D. melanogaster* (van Geffen et al., 2015b, Botha et al., 2017, McLay et al., 2018), which may either directly or indirectly affect subsequent fertilisation success and egg laying propensity (*sensu* Wolfner, 2002). Whatever the mechanism, the combination of reduced fecundity and longevity under ALAN is likely to have negative fitness implications for populations of invertebrates in lit urban environments.

The relationship between exposure to dim ALAN and resulting patterns of eclosion is novel could have arisen through variation in the timing of oviposition by females and/or due to developmental rate differences between offspring. ALAN induced disruption to the circadian timing of egg laying may have resulted in females exposed to 10 lx laying eggs earlier in the 18-hour oviposition window compared to 0 lx females. As eclosion in *D. melanogaster* is highly gated, with flies restricted to emerging in the hours surrounding dawn and requiring a critical development stage and size to be attained prior to eclosion (Myers, 2003), this could have resulted in fewer flies achieving the appropriate development stage and size for the first day eclosion gate. Alternatively, ALAN may have disrupted offspring development rate. This aligns with previous studies exploring invertebrate development in the presence of ALAN, although there is little consensus regarding directionality of this impact, with some species developing more rapidly (moth, *Mamestra brassicae* (van Geffen et al., 2014) and spider, *Eriophora biapicata* (Willmott et al., 2018)) while others exhibit slower development (black field cricket, *Teleogryllus commodus* (Durrant et al., 2018) and the amphipod, *Orchestoidea tuberculate* (Luarte et al., 2016)). The underlying mechanism promoting this variation is largely untested, however, eclosion differences could relate to ALAN-induced disruption to hormonal systems, such as release of prothoracicotropic hormone (PTTH) known to affect eclosion readiness and body size in *D. melanogaster* (McBrayer et al., 2007). Equally, foraging behaviour can vary under ALAN (Luarte et al., 2016) and developmental changes associated with size variation has been proposed as a possible mechanism in several previous studies (Durrant et al., 2018, Willmott et al., 2018). Therefore, ALAN-induced variation in foraging patterns may also explain why 10 lx flies were smaller than their 0 lx counterparts here. Finally, circadian disruption

could also weaken the eclosion gate due to ALAN's interaction with light-entrained core and peripheral clocks (Myers, 2003). Indeed, Thakurdas et al. (2009) reported that eclosion in *Drosophila jambulina* became arrhythmic under ALAN. We are unable to discriminate between the above theories although the fact 10 lx flies eclosing on day one were smaller than their 10 lx counterparts, provides notional support for the idea that fewer flies under 10 lx obtained the critical size for the first day eclosion gate. However, further investigation into the oviposition and eclosion timing under our model is needed to discriminate between these mutually non-exclusive alternatives.

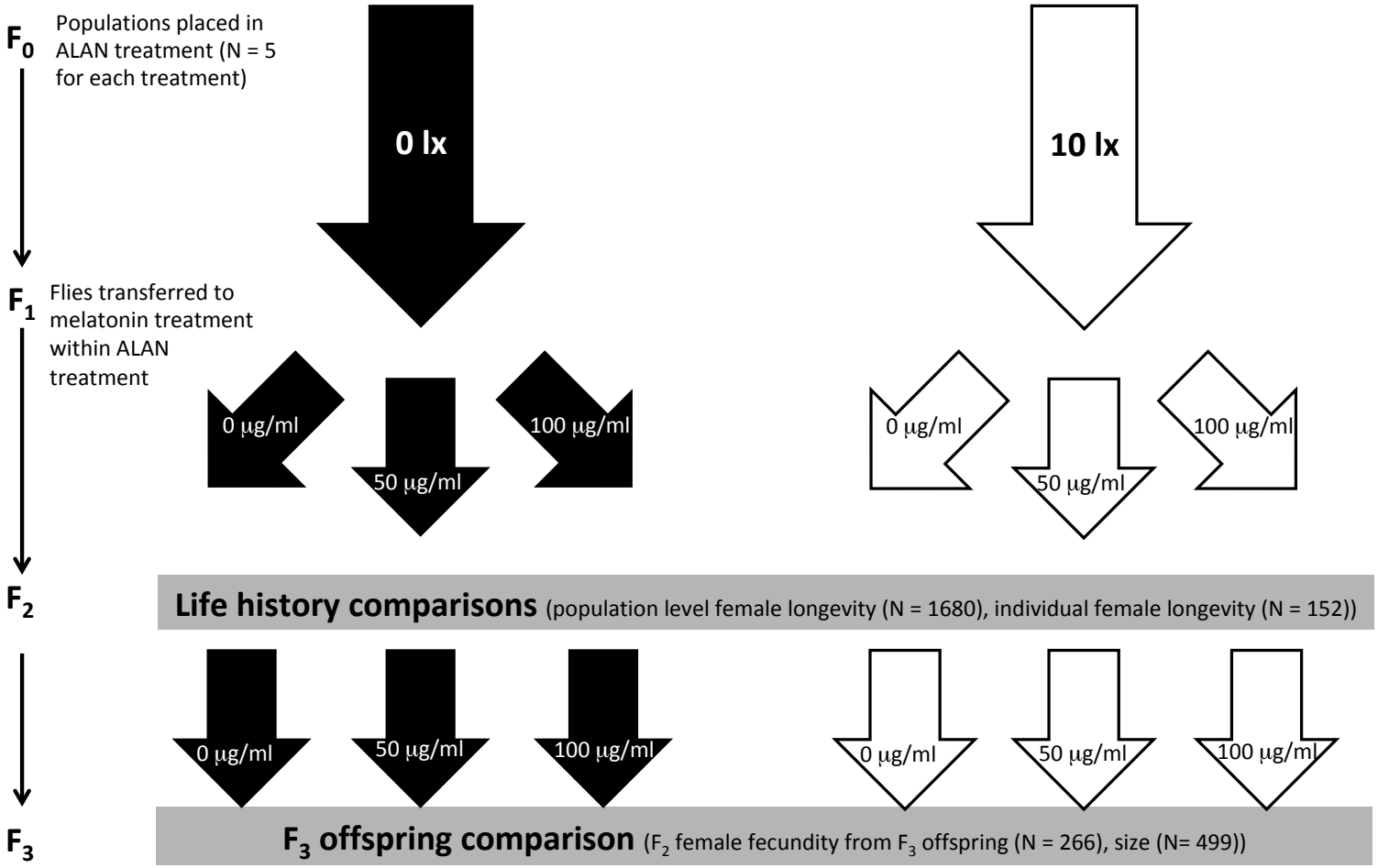
Contra to one of our central aims and despite replicating a previous study in dosage and administration regimen (Teran et al., 2012), we were unable to determine if melatonin is a driver behind the effects of ALAN. Instead, dietary melatonin supplementation appeared to have a toxic, rather than positive effect on the fitness of *D. melanogaster* in a high-density environment. A potential explanation for the observed decreased longevity under simultaneous melatonin and dark night treatments, involves melatonin's function as a chronobiotic: a constantly available high dose of dietary melatonin may overwhelm the natural peak of endogenously produced melatonin in flies maintained under dark nights, effectively making the flies somewhat arrhythmic with regard to activity/rest and causing a negative impact on longevity (Kumar et al., 2005). This reduced circadian rhythm could also explain why melatonin did not affect the longevity of flies under ALAN, which had comparable longevity to each other: if the relative peak of the melatonin cycle is already depressed by ALAN, then providing a constant supply of melatonin may not cause significant additional arrhythmicity. However, it should be noted that the highest dose of melatonin (100 μg/ml) in conjunction with ALAN, resulted in lower longevity compared to any of the dark night/melatonin - treatment combinations, indicating that in this model both melatonin and ALAN may act simultaneously as stressors, although not necessarily via the same pathway. Further investigation into the dosage, effects of the timing (i.e. diurnal/nocturnal/constant) and duration of melatonin supplementation is warranted to elucidate whether circadian desynchronisation could be a possible mechanism behind the interaction of ALAN's and melatonin's effects on longevity.

An alternative explanation for the negative effect of melatonin on longevity under dark nights in more stressful competitive environments, which could work alongside or independently to

the aforementioned effect on circadian rhythm, involves melatonin's antioxidant potential. There is a balance of oxidant versus antioxidant status in individual tissues that limits oxidative stress, generating 'optimal' ROS levels crucial for the normal cell signalling (Droge, 2002). Disruption to this status from high doses of dietary antioxidants has been shown to hinder cell function as well as causing potential toxicity due to a prooxidant effect (Bouayed and Bohn, 2010). Furthermore, early life exposure to low dose oxidants can increase longevity, due to adaptive protection mechanisms, such as mitohormesis (Obata et al., 2018). It is possible that supplementation with melatonin under dark nights, has disrupted normal cellular ROS levels and/or that dietary melatonin during the larval stage, has weakened a possible mitohormesis mechanism in our model.

In addition to effects on longevity, long-term supplementation of melatonin has consequences for development patterns, independent of ALAN's effect on development, with a higher proportion of flies eclosing on day one of eclosion with melatonin supplementation. One possible explanation for this, is melatonin's role in stimulating the release of PTTH. Higher PTTH would translate to faster development and smaller flies (McBrayer et al., 2007). The former was potentially evidenced here (if oviposition timing was unaffected), however we found no effect of melatonin on fly size. Consequently, we cannot state the mechanism behind melatonin's effect on development patterns with any certainty.

In conclusion, we have confirmed that ALAN negatively affects longevity, fecundity, size and for the first time, eclosion patterns (with respect to day of eclosion), in *D. melanogaster*. Additionally, we have demonstrated that melatonin is likely toxic to *D. melanogaster* at the concentrations or chronic administration regimens used here over total lifespan. This precludes us from unravelling melatonin's potential role as the driver of the phenotypic effects of ALAN, but it does highlight the extreme difficulty in isolating melatonin's many different biological functions and the relative contributions of its circadian and antioxidant capacities. Further research into melatonin supplementation under ALAN at different circadian doses, at different life stages and for different durations relative to lifespan is vital to help disentangle melatonin's many biological functions and understand the implications of ALAN in urban biological systems.



Supplementary Figure 5-1. Experimental design for melatonin and light treatment experimental comparisons

Chapter 6: General Discussion

The results presented in this dissertation demonstrate three key findings as outlined in Figure 6-1 and discussed in detail in this chapter for each of the text boxes. First, I establish that ALAN induces a phenotypic response in *D. melanogaster*. In **Chapter 2**, I show that *D. melanogaster* exhibits reduced oviposition following exposure to ALAN from as little as 1 lx (approximately three times full moon) and a reduction in longevity from exposure to 10 lx. I then demonstrate (through **Chapters 3 to 5**), further phenotypic shifts under ALAN of 10 lx, in mating behaviour, fecundity, size, changes to development patterns and lower ROS and oxidative damage levels, which together, are likely to be detrimental to individual fitness. These general results were replicated across chapters in different experiments and therefore, I can be confident that ALAN's effects on the behaviour and physiology of *D. melanogaster* are genuine. Second, I establish for the first time, that some of the observed phenotypic shifts in the presence of ALAN are sufficient to cause limited local adaptation in mating propensity in as few as 15 generations (**Chapter 4**). Finally, I demonstrate, contrary to my original prediction, that lifetime dietary supplementation with melatonin is potentially toxic and can negatively affect longevity and development times (**Chapter 5**). Consequently, this study was unable to determine melatonin's role in the effects of ALAN on life history traits.

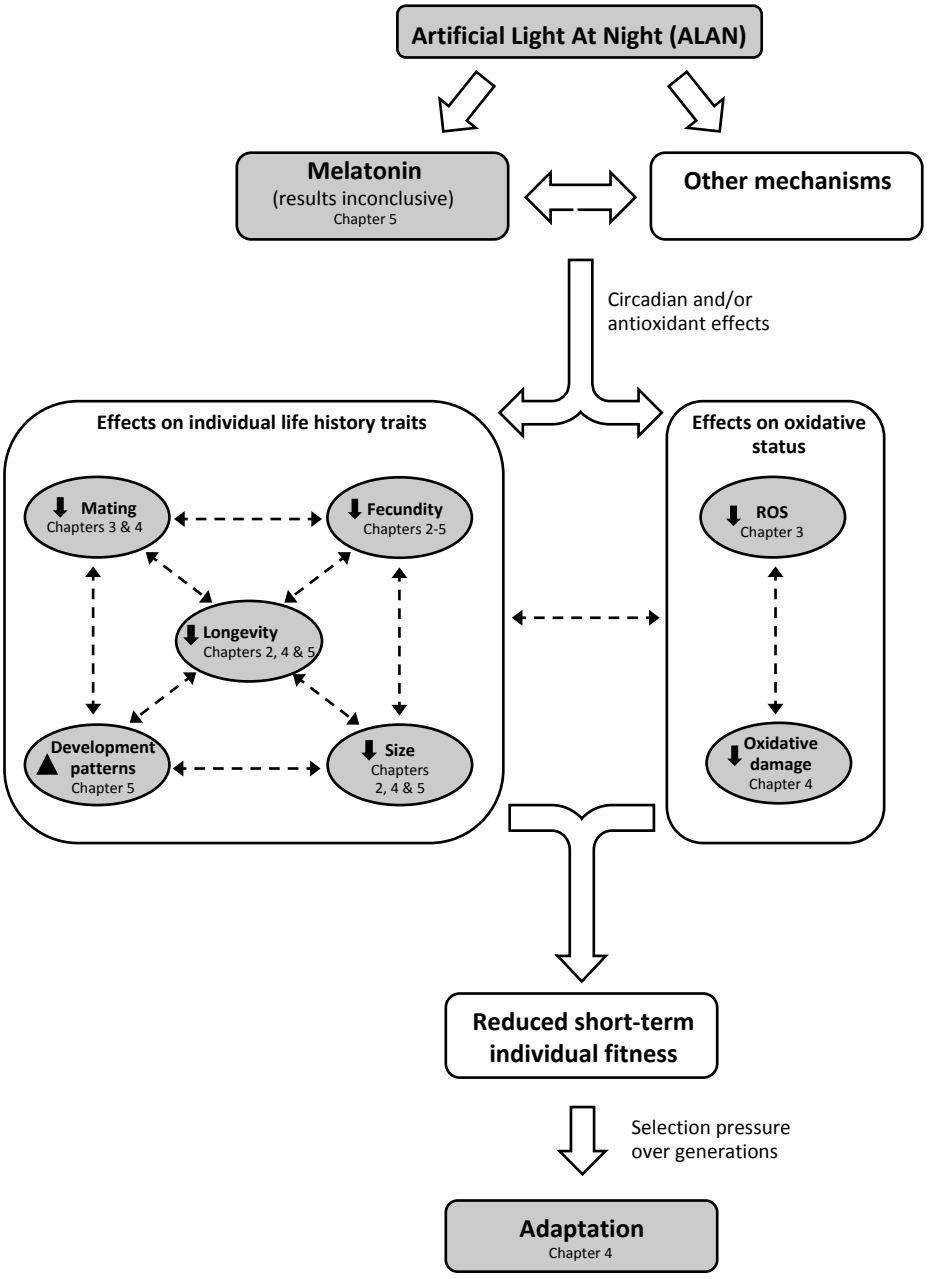


Figure 6-1 Schematic diagram of the effects, potential mechanisms behind and possible interactions between, the effects of artificial light at night (ALAN) in *Drosophila melanogaster*. Grey boxes depict demonstrated results in this thesis (and chapter numbers), black arrows indicate direction of change, black triangle indicates change only, white boxes show untested areas, white block arrows represent potential mechanisms and dotted lines show likely (but untested and inexhaustive) interactions. ALAN may reduce endogenous melatonin synthesis. Circadian and/or antioxidant disruption may be induced by melatonin and/or other un-investigated mechanisms to alter the life history traits of mating, fecundity, development patterns, size and longevity under ALAN, as well as decrease levels of reactive oxygen species (ROS) and oxidative DNA damage. These are likely to result in lower individual fitness and thus over generations, ALAN can act as a selective agent to induce adaptation.

6.1 ALAN affects phenotype

6.1.1 ALAN affects reproduction

I establish that ALAN (at 10 lx) disrupts mating propensity and mating behaviour in *D. melanogaster* in three different sets of experimental results (**Chapter 3** and baseline and evolved populations in **Chapter 4**). In all experiments ALAN lengthened the timing of at least one mating parameter and one result demonstrates that 20% fewer pairs under ALAN successfully mated than under dark nights. The increased time spent courting or copulating under ALAN in the field environment is likely to represent a fitness cost, due to increased competition and predation risk (Endler, 1987, Magnhagen, 1991) and may have knock-on effects on fecundity (Wolfner, 2002) (Figure 6-1). Indeed, I demonstrate that ALAN negatively affects female fecundity (**Chapters 2-5**). These studies explored several different measures of fecundity all of which were negatively impacted by ALAN except total eggs laid over a female's reproductive life. This latter measure however, was altered by ALAN in a curvilinear relationship with maternal age and cumulatively fewer eggs under ALAN were laid by middle life. Taken together, my results here indicate that ALAN affects mating and the likelihood, number and timing of offspring production. The reasons why ALAN might affect reproduction are likely due to indirect effects on hormonal systems which might affect pheromone production (Higgie et al., 2000), egg production (Howlader and Sharma, 2006) or via changes to antioxidant status (McLay et al., 2018). I propose that in a more competitive environment outside the laboratory (where females may die sooner), there are likely to be implications of ALAN on lifetime reproduction.

6.1.2 ALAN affects development patterns and size

The established correlation between female fecundity and size in *D. melanogaster* (Lefranc and Bundgaard, 2000), is possibly demonstrated here (Figure 6-1), with smaller flies developing under ALAN and fewer eggs laid under ALAN than under dark night treatment (**Chapters 2 and 4,** although size and oviposition were not measured simultaneously in individual flies). I demonstrate that early eclosing flies under ALAN are smaller in **Chapters 4 and 5**, however flies in **Chapter 2** did not differ in size between ALAN treatments. The differences between these results may have arisen because experimental flies used in **Chapter**

2 were the offspring of females that laid the most eggs and thus there was some potential selection for females better able to cope with ALAN. I hypothesise that differences in fly size under ALAN could potentially be linked to a direct influence of ALAN on foraging, but may also be due to indirect effects on hormones regulating growth, such as PTTH (McBrayer et al., 2007). I found no effect on average time to eclosion (**Chapter 2**), however the pattern of eclosion differed under ALAN (**Chapter 5**). This could be due to direct effects of ALAN on entrainment of the master clock, or by ALAN inducing later oviposition in females (if ALAN affects the timing of oviposition). However, it could be due to more indirect effects such as modulation of hormonal mechanisms such as PTTH (McBrayer et al., 2007). These potential linkages between fly size, eclosion patterns and oviposition timing, illustrate the complex interactions of the impacts of ALAN on life history traits (Figure 6-1). However, the effect of smaller size and later eclosing flies on individual fitness is uncertain and further research is required to understand their implications in the field environment.

6.1.3 ALAN effects longevity and survival

I report that while ALAN did not affect juvenile survival (**Chapters 2 and 3)**, ALAN did negatively impact adult longevity (**Chapters 2, 4 and 5**). Interestingly, females appear more sensitive to ALAN than males, with only female longevity affected in **Chapters 4 and 5**. This may reflect the cost of reproduction (Alonso-Alvarez et al., 2017) (Figure 6-1). However, in our model it appears that the effect of ALAN can override the usual observed trade-off between reproduction and longevity in *D. melanogaster* (Monaghan et al., 2009); although 10 lx females laid fewer eggs, they still died sooner than their 0 lx counterparts. At its greatest, there was a difference in longevity of approximately 15% of median life span and although in laboratory conditions it may not impact on individual female fitness, as females have largely ceased egg laying at this age, it is likely to be exacerbated in more stressful natural environments, where longevity is likely to be reduced, potentially encroaching on a female's fertile life-stage. I have proposed several mechanisms behind ALAN's effects on longevity which may operate simultaneously and include indirect impacts on circadian rhythmicity (Kumar et al., 2005), or trade-offs with systems dedicated to health maintenance, such as immune function (Moore and Siopes, 2000, Bedrosian et al., 2011b, Durrant et al., 2015) and potentially antioxidant capacity (McLay et al., 2018), following possible upregulation under ALAN.

6.1.4 ALAN effects oxidative status

One potential mechanism for reduced longevity is oxidative stress and damage (Figure 6-1), which is implicated as a major mechanistic driver for aging as well as in other trade-offs with life history traits (Harman, 1956, Dowling and Simmons, 2009). However, contrary to expectation, I demonstrate for the first time that ROS levels in pooled samples of ovaries (but not female heads) was lower under ALAN (**Chapter 3**). These results may explain some of the differences in female oviposition between ALAN treatments, as cell signalling is likely to be affected if ROS levels are below optimal levels (Droge, 2002). This result was mirrored in **Chapter 4** where oxidative DNA damage in pooled samples of female flies (but not males) was also lower under ALAN than in their 0 lx counterparts (Figure 6-1). At first examination, this does not support the theory of reduced-melatonin related increases in oxidative damage, however, there are currently no published studies demonstrating the full sequelae of this link under ecologically relevant ALAN intensities. I suggest that the lower oxidative damage and fecundity under 10 lx ALAN may be connected, demonstrating the potential cost of reproduction (Alonso-Alvarez et al., 2017). Alternatively, lower oxidative damage under ALAN may be connected to the potential upregulation of antioxidant capacity following a stressor (Monaghan et al., 2009). I assayed ROS and oxidative damage at one time point, therefore it is unknown whether these patterns persist throughout a female's life. I suggest supplementary research is required to investigate age specific oxidative damage in order to further understand the patterns and fitness implications of ALAN-correlated anomalies in oxidative status.

6.2 Potential mechanism

The mechanism driving of the effects of ALAN in *D. melanogaster* evidenced above, is likely not singular and probably involves different pathways acting in concert or potentially antagonistically. Additionally, as discussed above and shown in Figure 6-1, effects on one life history trait may have knock-on effects in others, resulting from direct effects, indirect effects or trade-offs between life history traits and oxidative status. However, I cannot state here with any certainty what these interactions are, but instead highlight that these links may be responsible for the effects evidenced in some life history and physiological traits and not ALAN directly. However, due to the replication of my results across experiments, I can state with

confidence that ALAN is either directly or indirectly the driver behind shifts in history traits and physiology in *D. melanogaster*.

The mechanisms through which life history traits and physiology are affected are likely to be both circadian and oxidative and may be epigenetic, as ALAN is linked to shifts in biological rhythms (Russart and Nelson, 2018) and I have demonstrated that oxidative status is affected (**Chapters 2 & 4**). However, I cannot disentangle them here, and it is likely that are acting together via different pathways. In Figure 6-1, I outline the life history and physiological changes observed under ALAN and that the interactions between these responses are uncertain and likely complex. It has been postulated that reductions in melatonin production is the causal pathway for the effects of ALAN (Reiter, 2002) (Figure 6-1), however, I was unable to test this mechanism in **Chapter 5**, as instead of inducing positive biological results, melatonin was detrimental to longevity under dark nights and hastened development under our supplementation regimen. My experiment used a constantly available dose of melatonin at doses previously shown to be beneficial in *D. melanogaster* (Izmaylov and Obukhova, 1999, Bonilla et al., 2002, Teran et al., 2012, Medina-Leendertz et al., 2014), so it is possible that a constant dose may have masked natural circadian rhythms (which could have already been disrupted under 10 lx ALAN). While my data can not provide evidence of melatonin' s role in the effects of ALAN, it does highlight that circadian rhythm is likely affected and that further research is required with different circadian dosing regimens and at different life-cycle stages to understand melatonin's potential relative contribution to the effects of ALAN via different pathways. Finally, I cannot exclude the possibility that other causal mechanisms are acting alone or with melatonin to produce the effects of ALAN evidenced in *D. melanogaster* (Figure 6-1). While not tested as part of this dissertation, ALAN can cause shifts in the master biological clock (Dubowy and Sehgal, 2017) that can be then relayed to tissues and organs via direct neuronal connections, independent to melatonin (Buijs et al., 2003). Thus, circadian shifts and hormone modulation can occur without melatonin as an intermediary. Further investigation into the expression of specific genes in peripheral clocks under ALAN in melatonin deficient mutants would be a potentail method to test this possibility.

6.3 Potential for adaptation

Together, my results of the negative effects of ALAN on key life history traits and physiology, suggest that ALAN may substantially reduce the fitness of individual flies (although the fitness effects of reduced oxidative damage is unknown) and should act as a selective pressure for local adaptation (Figure 6-1). In **Chapter 4**, my reciprocal transplant experiment demonstrated limited local adaptation in only one measure; improved mating propensity under 10 lx in flies that have evolved under 10 lx such that they were comparable to flies that had only experienced 0 lx at night. In our model, I would expect mating and oviposition to be the most likely traits to exhibit adaptation as they directly contribute to the next generation. The fact that oviposition did not vary between evolved and unevolved flies may be due to several reasons, including insufficient genetic variation in the founder population (unlikely as some adaptation was evidenced) or the strength of the selection pressure in relatively benign laboratory conditions under a limited number of generations of evolution. Regardless, of the limited extent of local adaptation, I have confirmed that an ecologically relevant intensity of ALAN has the potential to be a driver of evolutionary change.

6.4 Potential implications and future research directions

The results in this thesis establish numerous behavioural and physiological effects of ALAN in the model organism, *D. melanogaster* and add to the rapidly growing body of evidence demonstrating the negative implications of ALAN on organisms including humans (Gaston et al., 2014, Rich and Longcore, 2006). Humans share urban environments with a multitude of species, including invertebrates, that we largely rely on for pollination and other ecosystem services. Since the putative mechanism behind ALAN's effects is changes to endogenous melatonin (which is a highly evolutionarily conserved chemical, Tan et al., 2010), the effects of ALAN are likely to be taxonomically far reaching. If effects in other animals are similar to my results here, ALAN may have fundamental fitness consequences for individuals and populations in urban environments. Indeed, declines in pollinator communities are documented worldwide, and postulated to be due largely to anthropogenic environmental modification (Potts et al., 2016), including ALAN (Knop et al., 2017). Potentially, these impacts will have dire consequences for human food production. Consequently, there is a need for

modelling and theoretical studies to rapidly provide some predictions of effects of ALAN on populations and communities.

The pervasiveness of ALAN as an environmental pollutant is rapidly growing, with its extent increasing at 2.2% and radiance by 1.8% per annum globally (Kyba et al., 2017). Therefore, future research directions should be focused on mitigating the effects of ALAN on the increasing proportion of wildlife in its proximity, including in those areas experiencing relatively low intensity night lighting typical of skyglow. Fundamental to this approach is understanding the driving mechanism behind the behavioural and physiological shifts observed under ALAN, which would allow us to predict to some degree, species' responses. Potential avenues for future research aimed at investigating this mechanism and unravelling ALAN's circadian and antioxidant effects, could include genetic approaches using clock gene or melatonin knockouts as well as more direct measurements of melatonin and other endogenous antioxidants in relation to oxidative status in different organs and tissues. However, specific experiments to investigate the precise cause of some of the effects of ALAN evidenced in this thesis that are pivotal to population fitness, such as effects on reproduction, are also warranted. These approaches could include: pheromone assays and male song analysis, to understand effects on mating; and gamete and associated seminal fluid quality assays (perhaps in conjunction with endogenous melatonin manipulation) to assess consequences for fecundity. Additionally, as we know that ALAN's spectral properties is important in an organism's response to ALAN (Gaston et al., 2013), experiments under different wavelengths are likely to be paramount to the design of future sustainable urban lighting.

6.5 Concluding remarks

My thesis demonstrates the multiple negative effects of ecologically relevant ALAN intensity on life history traits and shifts in physiology in *D. melanogaster*. While these results cannot be directly extrapolated to other species, they are supported by other studies detailing similar negative effects (including in humans) and combined, should give us pause in the increasing illumination of our environment. I demonstrate that pinpointing the causal mechanisms behind these effects is complicated as there are likely many interactions and likely more than one mechanistic driver, however understanding some of the mechanisms will be important in

directing urban lighting strategies to mitigate ALAN's negative impacts. Finally, I look to the future and identify that ALAN can act as a selective agent to induce local adaptation under relatively benign laboratory conditions. Outside the laboratory, ALAN is likely to combine with other urban stressors to drive evolution in urban and peri-urban environments. Investigating the conditions under which this may occur will be paramount to the continued health of our cities.

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