University of Nevada, Reno

Circadian disruption in genes, brain, and behavior.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Neuroscience

By Cassandra Hui

Dissertation Advisor: Dr. Jenny Ouyang

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Advisor

Committee Member

Committee Member

Committee Member

Graduate School Representative

Markus Kemmelmeier, Ph.D., Dean
Graduate School

Abstract

Escalating urbanization and the ensuing proliferation of artificial light at night (ALAN) are profoundly altering the natural cycles of light and darkness that have long governed the behavior and biochemical processes of organisms. This disruption to circadian rhythms, our intrinsic biological clocks, poses significant health risks, including sleep disorders, metabolic dysfunctions, and increased susceptibility to chronic diseases. Understanding the mechanisms of circadian disruption and its broad implications is crucial, particularly as the modern world becomes increasingly illuminated. My dissertation delves into the effects of ALAN on circadian rhythms, employing *Drosophila melanogaster* (fruit flies) and *Taeniopygia guttata* (zebra finches) as model organisms to explore these impacts at molecular, neuronal, and behavioral levels. Chapter 1 introduces the conserved protein YIPPEE, likely involved in immune function, in *Drosophila* and shows downregulation of *Yippee* expression lengthens the circadian period and influences key clock genes. This suggests potential links between circadian systems and immune-related processes. Chapter 2 maps neuronal activation responses to ALAN in zebra finches through immediate early genes (IEGs) expression across 24 brain regions. These results uncover the differential activation of brain regions involved in vision, movement, learning, memory, pain processing, and hormone regulation under ALAN exposure. Chapter 3 shows that social interactions exacerbate ALAN-induced circadian disruptions. Birds housed together under ALAN experience

increased desynchronization of brain and liver timing and advanced activity onset, the first demonstration of social dynamics modulating the molecular clock in vertebrates.

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Introduction

In an era of rapid technological advances and escalating urban expansion, the universal rhythm of life—regulated by the cyclic dance of day and night—faces unprecedented disruption. Circadian rhythms, regulated by conserved biological clocks, orchestrate physiological and behavioral processes in virtually all organisms, from cyanobacteria to humans (*1*). Understanding disruptions and adaptations to this system is essential for human health.

Circadian rhythms regulate our sleep-wake cycle on a nearly 24-hour basis. The suprachiasmatic nucleus (SCN) in the brain acts as the master clock, synchronizing our biology with environmental light cycles (*2*). It directs peripheral clocks across the body's tissues and organs, harmonizing our internal timing. At the molecular level, a feedback loop involving essential proteins—Circadian Locomotor Output Cycles Kaput (CLOCK), Brain and Muscle ARNT-Like 1 (BMAL1), Cryptochrome (CRY), and Period (PER)—controls these rhythms (*3*). CLOCK and BMAL1 proteins promote transcription of *Cry* and *Per*, leading to the accumulation of CRY and PER proteins. These proteins then form complexes that inhibit their production by interacting with CLOCK/BMAL1 complexes, thus repressing their own transcription (*3*).

Modern life, characterized by non-stop activity and changing environmental conditions, pose significant challenges to maintaining circadian alignment. Circadian disruption, the misalignment between an organism's internal biological

clock and the external environment, leads to a host of physiological and behavioral dysregulations (*4*). This internal clock, an intricate network of genes and proteins, orchestrates the timing of countless bodily functions, from sleep-wake cycles and hormone secretion to metabolism and immune response (*4*). When this clock is thrown off balance, either by lifestyle factors such as shift work or external cues like light pollution, it can precipitate a cascade of adverse health effects. These include sleep disorders, metabolic syndromes, mood disturbances, and increased susceptibility to chronic diseases, underlining the critical role of circadian rhythms in maintaining health and homeostasis (*5, 6*). As society continues to grapple with these challenges, understanding and mitigating circadian disruption has emerged as a crucial area of research, with implications for public and ecological health, workplace policies, and individual lifestyle choices.

Artificial light at night (ALAN) has emerged as a significant circadian disruptor and is quickly becoming one of the most widespread global pollutants (*7*). The repercussions of ALAN exposure, while extensively documented across various species, have profound implications for human health and well-being (*8*). City life is especially susceptible. Nightshift workers, who represent an increasingly significant portion of the urban demographic, face a heightened risk of developing metabolic disorders, cardiovascular diseases, and certain types of cancer (*5, 9, 10*). Even at very dim levels, ALAN disrupts many facets of circadian regulation (*11-13*). As the night sky grows increasingly bright, understanding and mitigating the human-centric

impacts of ALAN is imperative. This situation underscores the urgent need for interdisciplinary research to tackle the challenges posed by this modern environmental issue.

To explore these questions, model organisms such as *Drosophila melanogaster* (fruit flies) and *Taeniopygia guttata* (zebra finches) provide invaluable insights. *D. melanogaster*, with its relatively simple yet highly conserved circadian machinery, offers a window into the fundamental workings of the circadian clock (*14, 15*). The genetic flexibility and well-mapped neural circuits of fruit flies make them an ideal system for dissecting the roles of circadian proteins and understanding how these rhythms are established, maintained, and disrupted (*16*). Their application in circadian research has been crucial in uncovering the molecular foundations of clock mechanisms (*17-20*).

Building upon the foundational insights gained from fruit flies, zebra finches offer a detailed view of circadian rhythms in vertebrates. They are social and diurnal, similar to humans, and serve as an excellent model for studying the effects of ALAN on complex physiological and behavioral responses (*21, 22*). Their human-like ability to process light information enables thorough investigations into how light pollution influences circadian regulation, especially in the context of social behaviors (*23*). This research sheds light on the wider consequences of ALAN exposure in vertebrates.

While there is a wide consensus that circadian disruption damages health, a comprehensive understanding of the intricate ways in which ALAN impacts these biological rhythms remains elusive. Particularly, the neuronal effects of ALAN exposure and how social interactions might mediate or exacerbate these disruptions are areas that have been less explored. Additionally, there is a lack of detailed knowledge on specific molecular disruptors potentially linked to other physiological functions, such as novel proteins like YIPPEE in circadian regulation and immune function, highlighting areas in need of further research.

This dissertation enhances our understanding of circadian rhythm disruption across molecular, neuronal, and behavioral level, utilizing both fruit flies and zebra finches to explore multifaceted functions. This work sheds light on the resilience and vulnerability of biological clocks to environmental disruptions. Through a series of studies that span from the molecular to the behavioral, this dissertation aims to bridge the gap between molecular mechanisms and ecological impacts, this work underscores the importance of maintaining circadian harmony in the face of environmental change.

Chapter Summaries

Chapter 1: YIPPEE Modulation of Circadian Rhythms in Drosophila melanogaster: Behavioral and Molecular Insights

This chapter highlights the critical role of circadian rhythms in various biological processes and their linkage to numerous health disorders when disrupted, including diabetes, sleep disorders, and certain cancers. Despite the extensive understanding of circadian rhythms' impact, the molecular mechanisms behind circadian outputs are not fully understood. The chapter introduces the protein YIPPEE, known for its zinc-binding domain and previous associations with the innate immune system (*24, 25*), and shows its involvement in the circadian rhythm. This connection indicates a potential link between circadian systems and immune-related processes, supported by YIPPEE's capability to modulate gene expression and interact with immunoglobulin-like proteins in insects, hinting at its role in complex regulatory networks. The study employs the GAL4 system and RNA interference in Drosophila melanogaster to downregulate YIPPEE expression (*26*), aiming to understand YIPPEE's influence on circadian regulation.

Chapter 2: Dim artificial light at night alters immediate early gene expression throughout the avian brain

ALAN affects physiological and behavioral processes, a significantly increasing problem as global urbanization expands (*27*). Despite ALAN's evident disruption of hormone regulation, immune function, and nighttime activity, its effects are not always coupled with canonical circadian genes that typically

synchronize behavior and physiology with natural photoperiods (*28, 29*). The precise mechanism by which dim ALAN influences neuronal activity and consequently disrupts downstream physiological and behavioral processes remains unclear, presenting a significant knowledge gap that impedes our ability to address light pollution effects effectively.

ALAN's influence extends to various brain regions, affecting hormone production, gene expression, neuronal survival, and plasticity, particularly in the hippocampus and caudal nidopallium (*28, 30*). In this chapter, I use immediate early genes (IEGs), cFos and ZENK, as markers for neuronal activation to map the brain's response to ALAN in *Taeniopygia guttata* (zebra finches).

Chapter 3: Birds of a feather flock together: light pollution's effects differ by social context.

The introduction of ALAN poses significant challenges by disrupting the circadian system's molecular, physiological, and behavioral rhythms, impacting overall health (*27*). Organisms rely on external cues like light and temperature to synchronize their biological rhythms with the day-night cycle, controlled by a feedback loop involving core circadian genes. The circadian clock, particularly the core clock in the SCN, orchestrates peripheral clocks in various tissues, influencing hormone secretion, activity periods, and more. Melatonin, produced at night by the pineal gland, is crucial for signaling sleep readiness and regulating biological functions, with its production closely regulated by the circadian clock. However,

ALAN disrupts behavioral rhythms, melatonin production, and various aspects of circadian regulation.

Previous research has largely focused on isolated animal models, overlooking the role of social interactions in circadian regulation, despite evidence suggesting that social environments significantly affect circadian rhythms (*31, 32*). For instance, social settings can synchronize activities and physiological processes among organisms, enhancing reproductive success and survival (*33*). Studies have shown that social interactions can align activity rhythms in groups and even restore rhythmic activity in arrhythmic environments (*18, 34*).

This chapter aims to explore how social conditions might alter the effects of ALAN, either mitigating or exacerbating its impact. I exposed zebra finches to ALAN under isolated and social conditions and compared their activity levels, circadian gene expression, and melatonin production with those in control dark conditions. I hypothesized that if social conditions provide circadian rescue, then circadian disruption, i.e., misalignment of gene-expression, physiology, and behavior with light-dark conditions, would be less in ALAN-exposed birds housed socially than in isolated conditions. Alternatively, if ALAN is a strong enough zeitgeber or stressor that the social context has no effect then exposure would elicit similar responses regardless of social condition.

References

1. U. Albrecht, H. Oster, The circadian clock and behavior. *Behavioural Brain Research* **125**, 89-91 (2001).
2. M. J. Zylka, L. P. Shearman, D. R. Weaver, S. M. Reppert, Three period homologs in mammals: Differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. *Neuron* **20**, 1103-1110 (1998).
3. J. C. Dunlap, Molecular bases for circadian clocks. *Cell* **96**, 271-290 (1999).
4. A. Patke, M. W. Young, S. Axelrod, Molecular mechanisms and physiological importance of circadian rhythms. *Nature Reviews Molecular Cell Biology* **21**, 67-84 (2020).
5. R. M. Lunn *et al.*, Health consequences of electric lighting practices in the modern world: A report on the National Toxicology Program's workshop on shift work at night, artificial light at night, and circadian disruption. *Science of the Total Environment* **607**, 1073-1084 (2017).
6. M. Schilperoort, P. C. N. Rensen, S. Kooijman, Time for Novel Strategies to Mitigate Cardiometabolic Risk in Shift Workers. *Trends in Endocrinology and Metabolism* **31**, 952-964 (2020).
7. C. C. M. Kyba *et al.*, Artificially lit surface of Earth at night increasing in radiance and extent. *Science Advances* **3**, (2017).
8. A. Deprato *et al.*, The Influence of Artificial Light at Night on Asthma and Allergy, Mental Health, and Cancer Outcomes: A Systematic Scoping Review Protocol. *International Journal of Environmental Research and Public Health* **19**, (2022).
9. D. B. Boivin, P. Boudreau, Impacts of shift work on sleep and circadian rhythms. *Pathologie Biologie* **62**, 292-301 (2014).
10. D. B. Boivin, P. Boudreau, A. Kosmadopoulos, Disturbance of the Circadian System in Shift Work and Its Health Impact. *Journal of Biological Rhythms* **37**, 3-28 (2022).
11. V. J. Alaasam *et al.*, Light at night disrupts nocturnal rest and elevates glucocorticoids at cool color temperatures. *Journal of Experimental Zoology Part a-Ecological and Integrative Physiology* **329**, 465-472 (2018).
12. J. Q. Ouyang *et al.*, Restless roosts: Light pollution affects behavior, sleep, and physiology in a free-living songbird. *Global Change Biology* **23**, 4987-4994 (2017).
13. D. M. Dominoni *et al.*, Integrated molecular and behavioural data reveal deep circadian disruption in response to artificial light at night in male Great tits (Parus major). *Scientific Reports* **12**, (2022).
14. C. Dubowy, A. Sehgal, Circadian Rhythms and Sleep in Drosophila melanogaster. *Genetics* **205**, 1373-1397 (2017).
15. M. W. Young, K. WagerSmith, L. Vosshall, L. Saez, M. P. Myers, Molecular anatomy of a light-sensitive circadian pacemaker in Drosophila. *Cold Spring Harbor Symposia on Quantitative Biology* **61**, 279-284 (1996).
16. M. N. Nitabach, P. H. Taghert, Organization of the Drosophila circadian control circuit. *Current Biology* **18**, R84-R93 (2008).
17. I. Edery, L. J. Zwiebel, M. E. Dembinska, M. Rosbash, TEMPORAL PHOSPHORYLATION OF THE DROSOPHILA PERIOD PROTEIN. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 2260-2264 (1994).

18. J. D. Levine, P. Funes, H. B. Dowse, J. C. Hall, Resetting the circadian clock by social experience in Drosophila melanogaster. *Science* **298**, 2010-2012 (2002).
19. S. A. Cyran *et al.*, vrille, Pdp1, and dClock form a second feedback loop in the Drosophila circadian clock. *Cell* **112**, 329-341 (2003).
20. S. R. Lone, V. K. Sharma, Social synchronization of circadian locomotor activity rhythm in the fruit fly Drosophila melanogaster. *Journal of Experimental Biology* **214**, 3742-3750 (2011).
21. V. M. Cassone, Avian circadian organization: A chorus of clocks. *Frontiers in Neuroendocrinology* **35**, 76-88 (2014).
22. A. Dawson, V. M. King, G. E. Bentley, G. F. Ball, Photoperiodic control of seasonality in birds. *Journal of Biological Rhythms* **16**, 365-380 (2001).
23. G. Wang, C. E. Harpole, A. K. Trivedi, V. M. Cassone, Circadian Regulation of Bird Song, Call, and Locomotor Behavior by Pineal Melatonin in the Zebra Finch. *Journal of Biological Rhythms* **27**, 145-155 (2012).
24. K. Roxstrom-Lindquist, I. Faye, The Drosophila gene Yippee reveals a novel family of putative zinc binding proteins highly conserved among eukaryotes. *Insect Molecular Biology* **10**, 77-86 (2001).
25. Y. Sun *et al.*, Gene expression patterns in response to pathogen challenge and interaction with hemolin suggest that the Yippee protein of Antheraea pernyi is involved in the innate immune response. *J Invertebr Pathol* **138**, 10-17 (2016).
26. J. B. Duffy, GAL4 system in Drosophila: A fly geneticist's Swiss army knife. *Genesis* **34**, 1-15 (2002).
27. D. M. Dominoni, R. J. Nelson, Artificial light at night as an environmental pollutant: An integrative approach across taxa, biological functions, and scientific disciplines. *Journal of Experimental Zoology Part a-Ecological and Integrative Physiology* **329**, 387-393 (2018).
28. V. J. Alaasam *et al.*, Effects of dim artificial light at night on locomotor activity, cardiovascular physiology, and circadian clock genes in a diurnal songbird. *Environmental Pollution* **282**, (2021).
29. K. Spoelstra, I. Verhagen, D. Meijer, M. E. Visser, Artificial light at night shifts daily activity patterns but not the internal clock in the great tit (Parus major). *Proceedings of the Royal Society B-Biological Sciences* **285**, (2018).
30. S. K. T. Taufique, A. Prabhat, V. Kumar, Constant light environment suppresses maturation and reduces complexity of new born neuron processes in the hippocampus and caudal nidopallium of a diurnal corvid: Implication for impairment of the learning and cognitive performance. *Neurobiology of Learning and Memory* **147**, 120-127 (2018).
31. G. Marimuthu, S. Rajan, M. K. Chandrashekaran, SOCIAL ENTRAINMENT OF THE CIRCADIAN-RHYTHM IN THE FLIGHT ACTIVITY OF THE MICROCHIROPTERAN BAT HIPPOSIDEROS-SPEORIS. *Behavioral Ecology and Sociobiology* **8**, 147-150 (1981).
32. N. Goel, T. M. Lee, Social cues accelerate reentrainment of circadian rhythms in diurnal female Octodon degus (Rodentia-Octodontidae). *Chronobiology International* **12**, 311-323 (1995).
33. O. Siehler, S. Wang, G. Bloch, Social synchronization of circadian rhythms with a focus on honeybees. *Philosophical Transactions of the Royal Society B-Biological Sciences* **376**, (2021).

34. N. A. Jha, V. Kumar, Female conspecifics restore rhythmic singing behaviour in arrhythmic male zebra finches. *Journal of Biosciences* **42**, 139-147 (2017).

Chapter 1: YIPPEE Modulation of Circadian Rhythms in Drosophila melanogaster: Behavioral and Molecular Insights

Abstract

The circadian rhythm is a fundamental biological process that governs physiological and behavioral functions in organisms. In Drosophila melanogaster, this intricate system relies on a negative feedback loop involving key transcriptional activators, CLOCK and CYCLE, which promote the transcription of critical clock genes, including *period* and *timeless*. Here, we investigate the role of a conserved protein, YIPPEE, previously associated with immune function, in the regulation of circadian rhythms. Through downregulation of YIPPEE, we observe a lengthening of the circadian period in constant darkness, accompanied by increased levels of the PERIOD protein. Further molecular analyses reveal elevated mRNA levels of *Clock*, *period*, and *timeless* genes, suggesting that YIPPEE may stimulate Clock transcription, thereby promoting the transcription of *period* and *timeless* genes. These findings shed light on the potential interplay between circadian regulation and immune-related processes, contributing to a deeper understanding of the molecular control of circadian rhythms.

Introduction

Time plays a pivotal role in shaping our society and structuring the world around us. Endogenous circadian rhythms serve as internal predictors of time, regulating physiological systems and behaviors in diverse species, including humans (*3*). These rhythms are driven by the coordinated activation and repression of clock genes, and organisms utilize external cues known as zeitgebers (ZT) to entrain their 24-hour internal rhythms. In the absence of these cues, a well-characterized negative transcriptional feedback loop involving core proteins operates to sustain the oscillations. In the fruit fly, *Drosophila melanogaster*, transcriptional activators, CLOCK (CLK) and CYCLE (CYC), form heterodimers to promote the transcription of repressor genes, *period* (*per*), and *timeless* (*tim*), leading to the rhythmic regulation of circadian processes (*4*). In the cytoplasm, PER and TIM form dimers that translocate back into the nucleus and block CLK/CYC activity, thus repressing their own transcription (*4*).

The importance of circadian rhythms in various biological processes has long been recognized, with extensive research demonstrating their influence on phenomena ranging from behavior to body temperature regulation (*1, 35, 36*). Disrupting the circadian rhythm has been linked to numerous health disorders, including diabetes, sleep disorders, and certain forms of cancer (*6, 37, 38*). However, despite extensive research, the precise molecular mechanisms governing circadian outputs remain elusive.

The protein YIPPEE, charatertized by a zinc-binding domain indicative of DNA binding and gene regulation, has been previously associated with the innate immune system (*25*). While limited information exists regarding the functions of YIPPEE, our study uncovers its involvement in circadian rhythms, suggesting a potential link between the circadian system and immune-related functions. The capacity of YIPPEE to modulate gene expression and interact with immunoglobulin-like proteins in insects also supports its potential role in complex regulatory networks.

Drosophila melanogaster, known for its clearly defined circadian neuron system, serves as an excellent model for circadian research. The circadian neurons are organized by anatomical location, which include ventrolateral neurons (LNvs), dorsal-lateral neurons (LNds), and three groups of dorsal neurons (DNs 1, 2, and 3) (*16*). Moreover, subgroups of lateral neurons, such as l-LNvs, s-LNvs, and 5th s-LNv, retain circadian rhythms through pigment dispersing factor (PDF) production (*14*). This well-established system allows targeted gene expression modulation through the GAL4 system. GAL4 is a gene regulating protein that can express interfering RNA to reduce gene expression in targeted cells (*26*).

In this study, we employ the GAL4 system to downregulate YIPPEE expression using RNA interference (RNAi) in Drosophila melanogaster. By examining the behavioral and molecular effects of YIPPEE downregulation on the circadian

rhythm, we aim to uncover insights into the potential role of YIPPEE in circadian regulation and its possible interplay with immune-related processes.

Methods

Fly Stocks

Flies were raised on a standard cornmeal and agar food at 25℃ with 12 hours of light and 12 hours of dark. The GAL4 lines used in this study were: *TD2* (*tim-GAL4, UAS-dicer2*), *PD2* (*Pdf-Gal4, UAS-dicer2*), *TD2; PG80* (*TD2; Pdf-GAL80*), *ED2* (*elav-GAL4; UAS-dicer2*). These lines were obtained from Vienna *Drosophila* RNAi Center (VDRC) and Bloomington *Drosophila* Center (BL).

Drosophila Activity Monitoring System

Flies 3-5 days old were individually placed in tubes in a Drosophila Activity Monitoring (DAM) device. This device contains infrared light that detects activity as the fly moves from one side of the tube to the other. The tubes had 2% agar and 5% sucrose food at one end and an air-permeable stopper at the other. The flies were left to entrain at a light-dark (LD) cycle of 12 hours each, starting at 9 a.m. and ending at 9 p.m. for 4 days. Light intensity was controlled around 500 lux. The flies were then switched to a dark-dark (DD) cycle for the next 7 days. Recorded locomotor activity was then filtered by the DAM system software in increments of 30 minutes. The individual monitoring tubes' DD data was analyzed by the FaasX (Fly Activity Analysis Suite) software.

Immunostaining

After entraining in a 12:12 LD cycle for 5 days, flies were fixed in 4% Paraformaldehyde for 20 minutes at room temperature and then dissected directly in a mixture of 1X Phosphate Buffered Saline (PBS) with 0.1% Triton (hereafter referred to as PT). After dissection, they were washed 3 times with PT, then 3 more times but this time for 10 minutes each. 500µl of Normal Goat Serum was added to each tube and left for 90 minutes. Brains were incubated with 250µl of primary antibody overnight at 4º C. The primary antibodies were anti-mouse PDF, 1:400 and rabbit PER, 1:1500. The next day the brains were washed with PT 5 times quickly and then 6 more times for 20 minutes on a rocker. A 1:200 dilution of the secondary antibodies were added to all tubes. They were then left for a second overnight incubation at 4º C, and the same sequence of PT washes was repeated. Finally, brains were washed with 1X PBS three times, then incubated in 80% Glycerol for 15 minutes, mounted on slides, and visualized on a Leica SP8 confocal microscope.

ImageJ Analysis

Pictures from the confocal imaging were visualized using NIH ImageJ software (*39*). The mean intensity of each neuron was measured after subtracting the mean of 3 background areas of equal size. The data from all brains of each genotype were then averaged and categorized by neuron type for quantification purposes. To compare the results between the different groups, t-tests were conducted.

RNA Extraction and qRT-PCR analysis

RNA was extracted from fly brains after being flash-frozen at the specified time points in the first day of DD after entraining in LD for 5 days. Fly heads were ground in TRIzol and mixed with chloroform. The tubes were then centrifuged, and the supernatant was left to mix with isopropanol overnight at -20℃. The RNA was then washed twice with ethanol. RNA quality was measured by a NanoDrop 1000 (Thermo Fisher Scientific, Massachusetts, USA).

Reverse transcription was completed by using a superscript III kit (Thermo Fisher Scientific, Massachusetts, USA). Quantitative real-time PCR (qRT-PCR) was subsequently performed using SYBR Master Mix (Thermo Fisher Scientific, Massachusetts, USA) with the following specific primers:

per forward primer (5'-GACTCGGCCTACTCGAACAG-3')

per reverse primer (5'-CGCGACTTATCCTTGTTGCG-3')

tim forward primer (5'-ATGGACTGGTTACTAGCAACTCC-3')

tim reverse primer (5'-GGTCCTCATAGGTGAGCTTGT-3')

Clk forward primer (5'-GCCTCGGAAACTATTACCTCCC-3')

Clk reverse primer (5'-CCATCTCATAGGCCAGGTCATA-3')

rpl32 forward primer (5'-CCGCTTCAAGGGACAGTATC-3')

rpl32 reverse primer (5'-ACGTTGTGCACCAGGAACTT-3')

yippee forward primer (5'-TCTTCAATTGCGCCCAATGCCAC-3')

yippee reverse primer (5'-ATGTGGCGACCCGTGAGCATGA-3')

Results

Downregulating YIPPEE lengthens the circadian activity period

We tested wheateher downregulating YIPPEE alters the flies' circadian period length using two RNAi lines with unique target locations on *Yippee* (Yippee^{RNAi-1} and Yippee^{RNAi-2}). We used a *tim-GAL4* to specifically reduce YIPPEE expression in circadian cells and enhanced the knockdown efficiency by co-expressing *UAS-dicer2*. Our results showed a significant lengthening of the circadian period by over an hour, 1.6 for Yippee^{RNAi-1} and 1.8 for Yippee^{RNAi-2} (Figure 1). Since both of our RNAi lines showed consitant results, we inferred that this was not due to off-target effects. To further refine our understanding, we limited the knockdown to PDF+ neurons with a *Pdf-GAL4* driver and produced similar results (Figure 1).

To determine the precise location YIPPEE is specifically required, we used a *tim-GAL4* with a *Pdf-GAL80*, which inhibits GAL4. Therefore, GAL4 and subsequently reduction of YIPPEE only occurred in PDF- circadian cells. This resulted in a sizable rescue of the period (Figure 1). Although still slightly lengthened over our control, these results imply that YIPPEE is prominently in the PDF+ neurons.

Additionally, we validated the downregulation of *Yippee* using qRT-PCR. *tim-GAL4* crosses showed only a minor reduction in *Yippee* expression. However, YIPPEE is widely expressed in the brain and targeting circadian neurons only might be insufficient. Consequently, we performed a pan-neuronal knockdown, *elav-GAL4*, which showed significant reduction of *Yippee* expression in the fly heads. This comprehensive approach confirmed the successful knockdown of *Yippee* and

its critical role in modulating circadian rhythms, particularly in PDF+ neurons (Figure 2).

YIPPEE affects the molecular clock

After determining YIPPEE plays a role in period length, we aimed to uncover the underlying mechanisms by examining its impact on PER expression, a key component of the molecular clock. We conducted immunostaining to analyze PER levels at their peak (ZT 1) in the small ventrolateral neurons (s-LNvs), revealing a notable increase in PER in the presence of *Yippee* knockdown mediated by *Pdf-GAL4* (Figure 3). This elevation could result from either enhanced transcription or increased protein stability. To further investigate, we focused on Yippee$^{RNAi\text{-}2}$, which demonstrated a more pronounced behavioral effect, to analyze its impact on core clock gene expression across six varied time points. The downregulation of YIPPEE led to increased levels of *per* mRNA (Figure 4A). Similarly, we found *tim* mRNA level also rose (Figure 4C) and *Clk* mRNA showed significant increases at all time points (Figure 4B). These findings suggest that downlregulating YIPPEE expression boosts the transcription of *per, tim, and Clk,* elucidating its integral role in regulating the molecular dynamics of the circadian clock.

Discussion

Our investigation sheds light on the involvement of the conserved protein YIPPEE in the regulation of circadian rhythms in *Drosophila melanogaster*. Through the downregulation of YIPPEE, we observed not only a lengthening of the circadian period but also increased expression of critical clock genes. This suggests YIPPEE's involvement in promoting transcription of *Clock*, and subsequently, *period* and *timeless*. These findings open new paths for investigating the intricate molecular mechanisms governing circadian rhythms and their potential links to immune-related processes, thereby contributing to our understanding of fundamental biological timing mechanisms.

Further examination revealed that suppressing YIPPEE in circadian-specific neurons led to elevated PER protein levels and an extended locomotor period under constant darkness. Concurrently, there was an upregulation of *Clk*, *per*, and *tim* mRNA, suggesting that YIPPEE might infulence gene expression, which is consistent with its zinc-binding domain which typically signifies a role in DNA interaction and transcriptional regulation (*24, 25*). These results imply that YIPPEE could be pivotal in maintaining circadian rhythm by modulating *Clk* in optimal transcription level, thereby promotes *per* and *tim* transcription. This proposes a mechanism whereby YIPPEE maintains rhythmicity by constraining *Clk* transcription to appropriate levels.

The broad sequence conservation of YIPPEE from insects to mammals suggests that it might have infulences beyond *Clk* (*24*), potentially impacting a range of cellular processes. Notably, ubiquitous downregulation of YIPPEE with actin-GAL4 was lethal, indicating its critical role in development and providing a notion at its extensive involvement in physiological regulation. Previous studies implicating YIPPEE in the altered expression of immune genes (*25*) underscore its significance in both immune function and now circadian rhythm regulation. Thus, YIPPEE appears to be a pivotal regulator of gene expression with dual roles in these processes. In light of these findings, there is a compelling need for deeper exploration into how YIPPEE intersects with circadian regulation and immune responses. Considering its structural similarities, it is plausible that YIPPEE might exhibit similar functions in mammals, providing a valuable model for understanding corresponding mechanisms in human circadian biology.

In conclusion, this research demonstrates that downregulating YIPPEE extends the circadian rhythm by upregulating core clock gene expression. These findings, combined with prior investigations, propose a potential link between the circadian rhythm and the regulation of the immune system. Although additional research is warranted for the precise mechanisms, YIPPEE YIPPEE offers a promising target of regulatory controls within biological systems.

Author contributions

Yong Zhang and Cassandra Hui designed the experiments. Cassandra Hui conducted the experiments, completed data analysis, and writing. Yong Zhang provided funding and oversite.

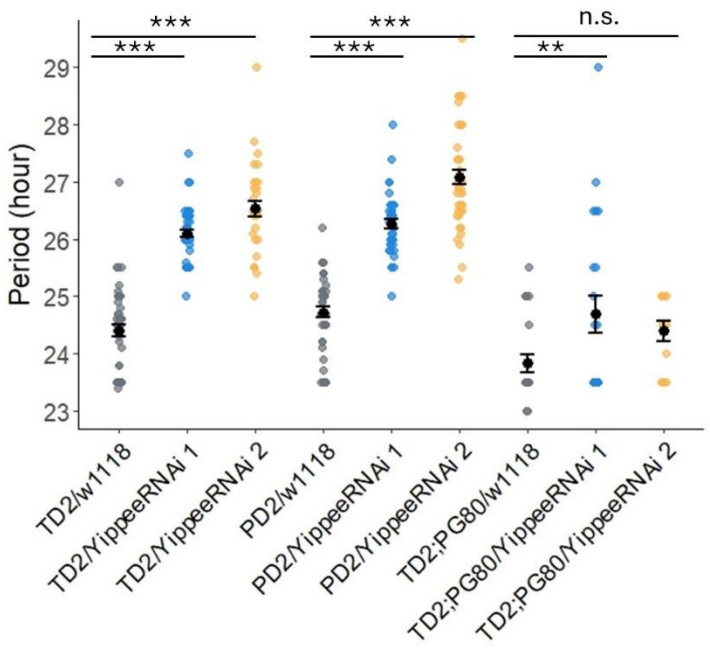

Figure 1. YIPPEE knockdown in TIM producing neurons shows a lengthening of the circadian rhythm, but not when expressed in PDF- neurons only. Crosses with a TIM and/or PDF driver were crossed with W^{1118} and two different *UAS-Yippee* RNAi lines, RNAi 1 and RNAi 2. *dicer2* was added for increased efficiency. YIPPEE knockdown with *tim* and *dicer2* (TD2) shows a period of 26-26.5 compared to controls at 24.4. YIPPEE knockdown with *Pdf* and *dicer2* (PD2) shows a period of 26-27 compared to controls at 24.5. YIPPEE knockdown with TD2, and *Pdf-GAL80* (TD2; PG80) shows a period of 24.2-24.6 compared to controls at 23.5. Data are reported as mean ± SE. Significance levels are $p > 0.05$ ns, $p \leq 0.05$ *, $p \leq 0.01$ **, and $p \leq 0.001$ ***.

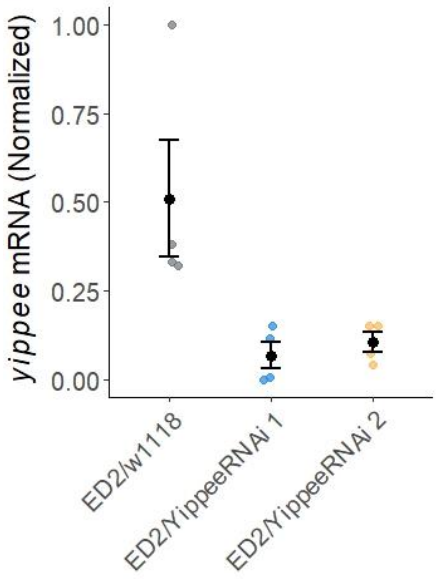

Figure 2. *elav-GAL4* knockdown shows a significant decrease in *Yippee*. Two different strains of *UAS-Yippee* RNAi showed a significant decrease in *Yippee* mRNA expression when knocked down with a pan-neuronal *elav-GAL4* and *UAS-dicer2* (ED2). Data are reported as mean ± SE.

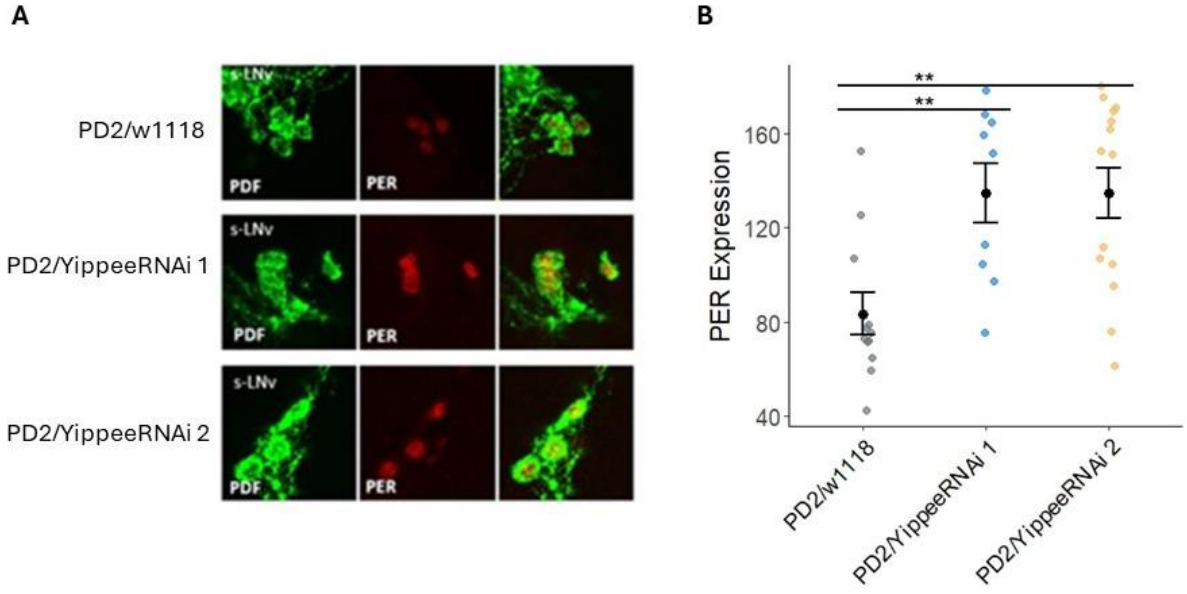

Figure 3. Knocking down YIPPEE increases PER expression in the s-LNvs. Immunostaining shows Period (PER) expression in the s-LNvs is increased when *Yippee* was knocked down with two different RNAi lines with *Pdf-Gal4, UAS-dicer2*

(PD2). Panel A shows representative staining for all three genotypes. Panel B shows the quantification of the data. Data are reported as mean ± SE. Significance levels are p > 0.05 ns, p ≤ 0.05 *, p ≤ 0.01 **, and p ≤ 0.001 ***.

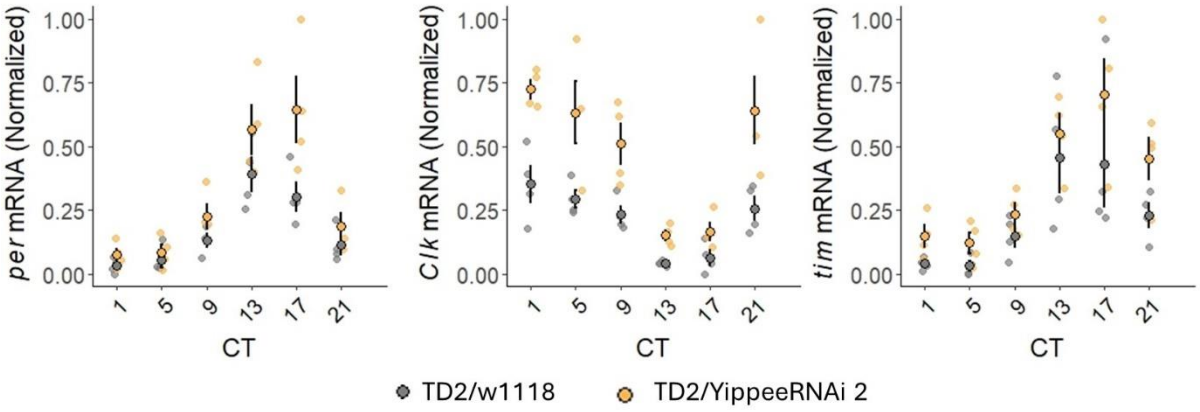

Figure 4. YIPPEE knockdown shows increased levels of *per*, *Clk*, and *tim* mRNA. *UAS-Yippee*$^{RNAi\ 2}$ was crossed with *tim-GAL4, UAS-dicer2* (TD2) and collected at time points CT 1, 5, 9, 13, 17, and 21. mRNA levels were analyzed through quantitative real-time PCR. YIPPEE downregulation shows an increase in *per, Clk,* and *tim* mRNA levels. Data are reported as mean ± SE.

References

1. J. C. Dunlap, Molecular bases for circadian clocks. *Cell* **96**, 271-290 (1999).
2. A. Patke, M. W. Young, S. Axelrod, Molecular mechanisms and physiological importance of circadian rhythms. *Nature Reviews Molecular Cell Biology* **21**, 67-84 (2020).
3. M. Schilperoort, P. C. N. Rensen, S. Kooijman, Time for Novel Strategies to Mitigate Cardiometabolic Risk in Shift Workers. *Trends in Endocrinology and Metabolism* **31**, 952-964 (2020).
4. A. Silva, M. J. dos Santos, D. L. G. Gitai, J. Coelho, T. G. de Andrade, Depression and anxiety symptoms correlate with diurnal preference, sleep habits, and Per3 VNTR polymorphism (rs57875989) in a non-clinical sample. *Journal of Affective Disorders* **277**, 260-270 (2020).
5. K. C. G. Van Dycke *et al.*, Chronically Alternating Light Cycles Increase Breast Cancer Risk in Mice. *Current Biology* **25**, 1932-1937 (2015).
6. Y. Sun *et al.*, Gene expression patterns in response to pathogen challenge and interaction with hemolin suggest that the Yippee protein of Antheraea pernyi is involved in the innate immune response. *J Invertebr Pathol* **138**, 10-17 (2016).
7. M. N. Nitabach, P. H. Taghert, Organization of the Drosophila circadian control circuit. *Current Biology* **18**, R84-R93 (2008).
8. C. Dubowy, A. Sehgal, Circadian Rhythms and Sleep in Drosophila melanogaster. *Genetics* **205**, 1373-1397 (2017).
9. J. B. Duffy, GAL4 system in Drosophila: A fly geneticist's Swiss army knife. *Genesis* **34**, 1-15 (2002).
10. K. Roxstrom-Lindquist, I. Faye, The Drosophila gene Yippee reveals a novel family of putative zinc binding proteins highly conserved among eukaryotes. *Insect Molecular Biology* **10**, 77-86 (2001).

Chapter 2: Dim artificial light at night alters immediate early gene expression throughout the avian brain (Published)

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Abstract

Artificial light at night (ALAN) is a pervasive pollutant that alters physiology and behavior. However, the underlying mechanisms triggering these alterations are unknown, as previous work shows that dim levels of ALAN may have a masking effect, bypassing the central clock. Light stimulates neuronal activity in numerous brain regions which could in turn activate downstream effectors regulating physiological response. In the present study, taking advantage of immediate early gene (IEG) expression as a proxy for neuronal activity, we determined the brain regions activated in response to ALAN. We exposed zebra finches to dim ALAN (1.5 lux) and analyzed 24 regions throughout the brain. We found that the overall expression of two different IEGs, cFos and ZENK, in birds exposed to ALAN were significantly different from birds inactive at night. Additionally, we found that ALAN-exposed birds had significantly different IEG expression from birds inactive at night and active during the day in several brain areas associated with vision, movement, learning and memory, pain processing, and hormone regulation. These results give

insight into the mechanistic pathways responding to ALAN that underlie downstream, well-documented behavioral and physiological changes.

Introduction

A continued rise in global urbanization also increases artificial light at night (ALAN), with light pollution now recognized as a disruptive pollutant (*27*). ALAN, even at dim levels, disrupts physiological and behavioral processes (*40*). However, these changes appear uncoupled from canonical circadian genes, which synchronize behavior and physiology to the natural photoperiod (*28, 29*), but see (*41*). Therefore, how dim ALAN affects neuronal activity to disrupt downstream physiological and behavioral processes remains unknown. This knowledge gap hinders our ability to predict and ameliorate responses to light pollution.

As ALAN disrupts hormone regulation, immune function, and nighttime activity, its effect could be linked to many corresponding brain regions (*11, 42*), especially if central circadian pacemakers are not disrupted. For example, ALAN disrupts melatonin and diurnal corticosterone production (*42*), which are produced by the adrenal and pineal glands and directly regulate the hypothalamus, septum, and hippocampus (*43-46*). ALAN also disrupts immune gene expression, neuronal survival, and plasticity in the hippocampus and caudal nidopallium (*42, 47-49*). Lastly, ALAN recruits new neurons to the medial striatum, theorized to replace dying neurons (*50*).

Neuronal activity induces immediate early gene (IEG) expression for new protein synthesis (*51, 52*). Therefore, IEGs indicate neuronal activation by associating firing with gene expression and have successfully been used to map neuronal pathways (*53, 54*). IEGs, such as cFos and ZENK, have been shown to respond to different stimuli. cFos expression is stimulated by cAMP and calcium, and ZENK expression by injury, stress, etc. (*55-57*). Using both IEGs can generate a holistic, detailed map of brain activity for a more representative analysis (*54, 58*).

We analyzed ALAN's impact on IEG expression throughout the whole brain of Zebra finches (*Taeniopygia guttata*), an excellent diurnal model organism, as they translate external light similarly to most vertebrates (*59, 60*). Since ALAN initiates nighttime activity, we predicted activation in the visual and motor pathways, but that these areas would be similar to birds awake during the day. We also predicted, based on previous research, activation in areas involved in learning and memory, particularly the hippocampus, caudal nidopallium, and striatum (*48, 50*). We found that ALAN significantly altered IEG expression of cFos and ZENK in the hyperpallium, mesopallium, nidopallium, para-hippocampalis, striatum, entopallium, arcopallium, hippocampus, and septum compared to day and/or night birds.

Methods

Experimental Design

Thirteen male zebra finches (~100 days old) were kept in outdoor aviaries at the University of Nevada, Reno with no previous exposure to ALAN. When they were ~140 days old, we moved them to individually housed indoor 47 x 31 x 36cm cages and entrained them to 12 hours light and 12 hours dark (12L:12D) for four weeks. For daylight, we used 1.4-Watt 5000 K light emitting diode (LED) rated at 95 Lumens lights at 0:00 (zeitgeber time (ZT) 0) and lights off at 12:00 (ZT 12). Birds were given food and water *ad libitum*. Each cage contained a mechanized perch that relayed hop activity to MATLAB every minute. Cages had individual light-occlusion shades and constant white noise in the background to limit visual and acoustic cues.

We video recorded 30mins of behavior 90 mins before perfusion and activity via automated perches (*11*). An observer blind to the treatments determined time spent eating/drinking, grooming, hopping, or no movement for the video recordings. We conducted a power analysis based on previously collected behavior data from control and ALAN exposed birds and determined that at least 3 birds were needed per treatment group (Power=0.8, α=0.05, effect size=2, number of groups=3).

Birds were randomly assigned to one of 3 conditions: control night (12 hours light: 12 hours dark; 12L:12D sacrificed at dark night: ZT 14, n = 4), control day (12L:12D sacrificed at day: ZT 10, n= 4), and experimental ALAN (12 hours light: 12 hours dim light; 12L:12Ldim sacrificed at night with artificial light: ZT 14, n = 5). We chose the control day timepoint as close as possible to the night timepoints to be certain in capturing awake birds but also avoiding larger differences in circadian

activity. As determined by One-Way ANOVA, groups did not differ in initial mass ($p = 0.62$). After the 4-week entertainment period, we sacrificed the control night group during the dark period (ZT 14) and the control day group during the light period (ZT 10). We sacrificed individuals in the ALAN group 2 hours after the bird's 1st exposure to ALAN (ZT 14), to obtain peak protein expression and avoid overlap from the light period. ALAN was standardized to around 1.5 lux ± 0.01 from a 20 x 1.5 cm 5000 K broad spectrum LED strip. This was done with an Extech Easyview Digital Light Meter (model EA13) and lux was calculated using a mean measurement at perch height and two opposing base corners. For a full-spectrum description of the lights, please see (*28*).

Immunohistochemistry

We anesthetized birds with 0.1 ml of anesthesia made from 30mg Ketamine HCl, 105mg Xylazine, and 8.25 ml saline. After no response to a hard toe pinch, weight was taken, and we perfused birds with 1X PBS for five minutes and 4% paraformaldehyde in 1X PBS (PFA) for 13 minutes. Brains were removed, left in 4% PFA for 24 hours, switched to a 15% sucrose solution for 4 to 12 hours, followed by a 30% sucrose solution overnight, and then flash frozen with powdered dry ice and stored in -80°C until slicing.

We cut the left hemisphere of the brain sagittally at 45 µm thickness in six series. Series 1 was stained for imaging (total slices analyzed: ALAN = 90, control day = 67, control night = 67) and 2-6 were stored in cryoprotectant (3.3% sucrose, 0.01% Polyvinyl-pyrrolidone (PVP-40), 30% ethylene glycol in 0.1M Phosphate Buffer) in -80° C.

We incubated brain slices in a blocking solution (4% BSA, 0.4% triton, 0.05% Na-Azide in 1x PBS) for 3 hours and then with primary antibody diluted in blocking solution (c-Fos anti-rabbit polyclonal from ABCAM (ab190289) diluted 1:1000, ZENK anti-mouse monoclonal received from Dr. Keays' lab (*58*) diluted 1:300) in 4° C for ~46 hours. We washed slices 3 times in 1X PBS for 25 mins each at room temp and incubated overnight at 4° C with secondary antibodies (anti-rabbit 488 (from ABCAM ab150081) diluted 1:1000 and anti-mouse 594 (from ABCAM ab150116) diluted 1:1000), protected from light. We then incubated slices with DAPI for 15-25 mins at room temperature and washed them in 1X PBS 3 times. Slices were mounted with antifade mounting medium (VECTASHIELD ®) on slides. We imaged tile scans of full slices within 1 week of mounting on a Leica TCS SP8 confocal microscope.

Statistical Analyses

We analyzed images on ImageJ. We determined brain regions using anatomical locations with DAPI staining and a reference atlas from

zebrafinchatlas.org. Cells were determined positive for cFos or ZENK if they were three times the mean brightness and overlapped with DAPI. We divided the number of positive IEG cells by the total DAPI cell count to determine expression percentage in representative areas measured over several slices.

We performed statistical analyses in R, version 4.1.2 (R Development Core Team 2011). We ran generalized linear mixed-effect models to assess if IEG expression levels were affected by the treatment group as a fixed effect (*lme4* package). Slice number and bird ID were included as random effects. We used a Kruskal-Wallis test to analyze the interaction of behaviors and treatment groups. We ran a correlation matrix for all brain regions in each treatment for both cFos and ZENK (Figure S1).

Ethics Statement

All procedures were conducted in accordance with the National Institute of Health Ethical Use of Animals and approved by the University of Nevada, Reno Institutional Animal Care and Use Committee.

Results

Activity

There was a significant difference in the interaction between behavior and treatment group (Kruskal-Wallis test: Grooming: chi-squared = 6.22, p= 0.05, Feeding: chi-squared = 11.51, p< 0.01, Hopping: chi-squared = 6.45, p=0.04, Inactive: chi-squared = 7.20, p=0.03). Hop activity measured via perch recordings also showed that birds had significantly lower nocturnal activity (control night) than daytime activity (control day) and nocturnal activity under ALAN (Kruskal-Wallis test: chi-squared = 6.47, p=0.04, Figure 1).

IEG Expression

The ALAN group was significantly different from the control night (cFos p = 0.027, ZENK: p = 0.037) but not the control day (cFos: p = 0.17, ZENK: p = 0.66) when all 24 brain regions were analyzed together (Figure 2A). We broke down the analysis by looking into two major pathways—motor and visual—as well as additional areas. There was no significant difference between cFos and ZENK expression between the ALAN group and either control in all combined areas analyzed in the motor pathway (cFos-Day: p = 0.40, cFos-Night: p = 0.14, ZENK-Day: p = 0.72, ZENK-Night: p = 0.14). Similarly, we saw no significant difference for all areas analyzed in the visual pathway (cFos-Day: p = 0.08, cFos-Night: p = 0.07, ZENK-Day: p = 0.61, ZENK-Night: p = 0.11). However, individual areas in both pathways were significantly different (Table 1; Figures S2 and S3).

To determine if the expression was based on activity, we reanalyzed expression with birds separated into only two groups of active (n = 7) or inactive (n =

6; total minutes of activity <1 minute) 90 minutes before perfusion. Active birds included the control day group and non-active included the control night, with the ALAN group split between the two, based on activity. There was no significant difference in cFos or ZENK expression overall between active and non-active birds (cFos: $z = 1.18$, $p = 0.24$, ZENK: $z = 1.70$, $p = 0.09$). Additionally, there was no significant difference between active and non-active birds in the whole motor (cFos: $z = 1.28$, $p = 0.20$, ZENK: $z = 1.81$, $p = 0.07$) or visual (cFos: z = 0.65, $p = 0.51$, ZENK: $z = 1.37$, $p = 0.17$) pathways.

In the visual pathway, the ALAN group showed significantly higher cFos expression in the striatum adjacent to the core of the entopallium, posterior hyperpallium (Figure 2B), and ventral mesopallium adjacent to the core of the entopallium than the control night group, and significantly higher ZENK expression in the nidopallium adjacent to the core of the entopallium but lower in the core of the entopallium. The ALAN group also showed significantly higher cFos expression than the control day group in the striatum adjacent to the core of the entopallium and posterior hyperpallium areas (Table 1).

In the motor pathway, the ALAN group showed significantly higher cFos expression than the control night group in the anterior mesopallium dorsal (Figure 2B) and anterior mesopallium ventral regions and significantly higher ZENK expression in the anterior mesopallium dorsal and nidopallium caudolateral regions (Figure 3). The ALAN group also had significantly higher levels of cFos expression

compared to the control day group in the anterior mesopallium dorsal and lateral int arcopallium and higher ZENK expression in the nidopallium caudolateral. However, the ALAN group had significantly lower ZENK expression in the anterior striatum, nidopallium adjacent to the basorostral nucleus, and ventral mesopallium adjacent to the basorostral nucleus (Table 1). There was no significant difference between active and non-active birds in the anterior mesopallium dorsal, nidopallium caudolateral, or nidopallium adjacent to the basorostral nucleus.

The ALAN group also showed higher cFos expression in the area parahippocampalis, medial dorsal mesopallium, entopallium (Figure 2B), and lateral ventral mesopallium and higher expression of ZENK in the caudal striatum, medial dorsal mesopallium (Figure 2C), entopallium (Figure 2C), and lateral ventral mesopallium as compared to the control night group, but lower levels of ZENK expression in the hippocampus (Figure 2C) and septum. The ALAN group also showed higher levels of cFos expression in the entopallium and higher ZENK expression in the area para-hippocampalis, medial dorsal mesopallium, and entopallium as compared to the control day group (Table 1).

Discussion

Although ALAN is a pervasive pollutant, the neuronal response remains unclear. We imaged IEG expression of 24 brain regions during the day, night, and ALAN exposure in birds and found various regions were significantly differentially activated among the treatment groups. Overall, ALAN-treated birds were more like

control-day birds in total IEG expression. However, six brain regions differed among all three treatment groups: anterior mesopallium dorsal, entopallium, medial dorsal mesopallium, posterior hyperpallium, nidopallium caudolateral, and striatum adjacent to the core of the entopallium.

Vision

In the visual pathway, control night birds (LD sacrificed during the night) were significantly different from control day (LD sacrificed during the day) and ALAN birds (LLdim sacrificed during the night). These large differences are to be expected as LD control night birds were inactive. However, we still found two areas had significantly stronger cFos expression for ALAN birds than both control groups: posterior hyperpallium and striatum adjacent to the core of the entopallium. ALAN was a novel visual stimulus for the birds, likely employing a visual neuronal response.

The entopallium, the most prominent area to emerge, was significantly different from both controls and both IEGs. The entopallium is involved in visual pattern recognition (*61, 62*). Surprisingly we found that birds exposed to ALAN had different IEG expression in visual pathways compared to day controls. We see that even very dim levels of ALAN (around 1.5 lux) elicit a clear response in recognizing this visual input.

Movement

Out of the seven regions of the motor pathway analyzed, ALAN birds were significantly different from the day controls in either cFos or ZENK in six of them. However, when accounting for activity, the ALAN group remained significantly different with increased expression in the anterior mesopallium dorsal and nidopallium caudolateral and decreased in the nidopallium adjacent to the basorostral nucleus. Although the nidopallium caudolateral has additional functions, the anterior mesopallium dorsal and nidopallium adjacent to the basorostral nucleus are differentially activated under ALAN and not associated with hopping. These areas may be picking up movement we did not track, such as head turns and flapping wings, or associated with other functions we are unaware of.

Memory and Learning

We found birds exposed to ALAN were significantly different from both controls in areas associated with learning and memory. The ALAN group had significantly higher IEG expression than the day and night controls in the area para-hippocampalis and medial dorsal mesopallium, which are involved in spatial and object recognition and associative learning respectively (*63, 64*). The ALAN birds also had significantly lower IEG expression than the night controls in the hippocampus, which is involved in spatial memory and learning (*65, 66*). Dim ALAN

dampens behavioral measures of learning and memory which have also been correlated with structural alterations in the hippocampus (*48, 67, 68*). Lower nocturnal IEG expression in the hippocampus may partially explain why dim ALAN suppresses gene expression in the hippocampus (*48, 68*). It is believed that sleeping activates the hippocampus for memory consolidation (*69*). Indeed, we see higher IEG expression in our control night birds than day. A nocturnal suppression of hippocampal activity may impair memory consolidation and learning under ALAN.

ALAN treatment birds had significantly higher IEG expression in the nidopallium caudolateral than either of the controls. This aligns with previous research that has found dim ALAN alters the neuroarchitecture of the nidopallium caudolateral, the avian equivalent of the prefrontal cortex (*48, 70, 71*). The nidopallium caudolateral has been implemented in mimicking prefrontal area structures by having the same receptor architecture as the Brodmann Area 10 in humans, which is involved in many processes including reward and conflict, working memory, and pain (*72, 73*). IEG activation in areas associated with memory support previous findings that ALAN impairs learning and memory (*67*). Additionally, the avian nidopallium caudolateral along with the entopallium have been shown to display attentional mechanisms (*74*), implying an alert state in our ALAN exposed birds.

Pain Processing

Another association to emerge was pain processing. Dim ALAN has been shown to alter pain reception in mice (*75*). ALAN treatment birds had significantly higher activity in the caudal striatum from night controls and significantly higher activity in the nidopallium caudolateral from both controls. Although not much is known about the avian caudal striatum, this area is related to anxiety and pain in mice (*76*). Additionally, the nidopallium caudolateral has been associated with the Brodmann Area 10 in humans, also involved in pain reception (*72, 73*).

Hormone Regulation

Birds under ALAN had significantly decreased IEG expression compared to the control night birds in the septum and hippocampus, which directly regulate hormones leading to downstream physiological changes (*77*). The hippocampal-septal pathway regulates hormones involved in stress and immune function including; corticotropin-releasing hormone (*78, 79*), thyrotropin-releasing hormone (*80, 81*), and corticosterone (*82, 83*).

Our results show that ALAN typically increases IEG expression in differentially activated areas compared to both controls. However, reduction of ZENK expression in the septum and hippocampus implies reduced neuronal activation in co-regulated functions—such as hormonal control. This is supported by previous research that ALAN alters hormone production (*40, 84*).

In summary, through fine analyses of IEG expression, we found that initial ALAN exposure activates brain areas involved in vision, movement, learning and memory, pain processing, and hormone regulation, which may be differentially regulated under prolonged sleep loss or long-term exposure to ALAN. Additionally, first time exposure to ALAN at a different time in the night may produce differential responses from those we observed. Although ALAN may not be eliciting changes through circadian regulation, we still see substantial responses across brain areas that warrant further study. ALAN creates a unique brain state that is significantly different from day or nighttime brain activity. Dim light creates a novel environment, different from birds active in the day or sleeping at night, which produced widespread differential brain activity.

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Author contributions

CH, VA, and JO designed the experiments. CH, NC, and AC conducted the experiment and completed data analysis. SP and JO oversaw the project, provided training, and reviewed analyses. JO, NC, and AC provided funding. All authors contributed to and reviewed the writing.

Table 1. Analysis of IEG expression, arranged alphabetically by region, for individual brain regions comparing control night and control day groups against birds exposed to ALAN.

Brain Area	**Pathway**	**cFos Significance**	**cFos Z Value**	**ZENK Significance**	**ZENK Z Value**
Anterior Hyperpallium	Motor	ALAN = Night (p= 0.076)	-1.65	ALAN = Night (p= 0.189)	-1.31
		ALAN = Day (p= 0.099)	-1.78	ALAN = Day (p= 0.752)	-0.32
Anterior Mesopallium Dorsal	Motor	ALAN ≠ Night **(p= 0.039)**	-2.07	ALAN ≠ Night **(p= 0.017)**	-2.40
		ALAN ≠ Day **(p= 0.008)**	-2.64	ALAN = Day (p= 0.145)	-1.46
Anterior Mesopallium Ventral	Motor	ALAN ≠ Night **(p= 0.018)**	-2.36	ALAN = Night (p= 0.154)	-1.43
		ALAN = Day (p= 0.898)	0.13	ALAN = Day (p= 0.858)	-0.18
Anterior Nidopallium	Motor	ALAN = Night (p= 0.219)	-1.23	ALAN = Night (p= 0.414)	-0.82
		ALAN = Day (p= 0.801)	-0.25	ALAN = Day (p= 0.982)	0.02
Area Para-hippocampalis		ALAN ≠ Night **(p= 0.026)**	-2.23	ALAN = Night (p= 0.090)	-1.70
		ALAN = Day (p= 0.712)	-0.37	ALAN ≠ Day **(p= 0.005)**	-2.79
Anterior Striatum	Motor	ALAN = Night (p= 0.749)	-0.32	ALAN = Night (p= 0.608)	0.514
		ALAN = Day (p= 0.750)	0.32	ALAN ≠ Day **(p= 0.017)**	2.39
Caudal Striatum		ALAN = Night (p= 0.053)	-1.94	ALAN ≠ Night **(p= 0.031)**	-2.15
		ALAN = Day (p= 0.129)	-1.52	ALAN = Day (p= 0.325)	-0.99

Dorsal Lateral Nidopallium	Motor	ALAN = Night (p= 0.401)	0.84	ALAN = Night (p= 0.943)	0.07
		ALAN = Day (p= 0.362)	0.91	ALAN = Day (p= 0.436)	0.78
Entopallium		ALAN ≠ Night **(p< 0.001)**	-137.6	ALAN ≠ Night **(p< 0.0001)**	-4.79
		ALAN ≠ Day **(p < 0.001)**	-195.1	ALAN ≠ Day **(p= 0.014)**	-2.46
The core of the Entopallium	Visual	ALAN = Night (p= 0.010)	1.65	ALAN ≠ Night **(p= 0.038)**	2.08
		ALAN = Day (p= 0.058)	1.90	ALAN = Day (p= 0.438)	0.78
Hippocampus		ALAN = Night (p= 0.251)	1.15	ALAN ≠ Night **(p= 0.040)**	2.06
		ALAN = Day (p= 0.953)	-0.06	ALAN = Day (p= 0.791)	0.27
Lateral Int Arcopallium	Motor	ALAN = Night (p= 0.983)	-0.02	ALAN = Night (p= 0.394)	0.85
		ALAN ≠ Day **(p= 0.030)**	-2.17	ALAN = Day (p= 0.448)	-0.76
Lateral Ventral Mesopallium		ALAN ≠ Night **(p= 0.001)**	-3.24	ALAN ≠ Night **(p= 0.002)**	-3.05
		ALAN = Day (p= 0.109)	-1.60	ALAN = Day (p= 0.554)	-0.59
Medial Dorsal Mesopallium		ALAN ≠ Night **(p= 0.014)**	-2.47	ALAN ≠ Night **(p= 0.001)**	-3.33
		ALAN = Day (p=0.206)	-1.27	ALAN ≠ Day **(p< 0.0001)**	-3.98
Ventral Mesopallium adjacent to the Basorostral Nucleus	Motor	ALAN = Night (p= 0.746)	-0.32	ALAN = Night (p= 0.231)	-1.20
		ALAN = Day (p= 0.667)	0.43	ALAN ≠ Day **(p< 0.0001)**	2.87
Ventral Mesopallium adjacent to the Core of the Entopallium	Visual	ALAN ≠ Night **(p= 0.014)**	-2.47	ALAN = Night (p= 0.175)	-1.36
		ALAN = Day (p= 0.879)	-0.15	ALAN = Day (p= 0.590)	0.54

Nidopallium adjacent to the Basorostral Nucleus	Motor	ALAN = Night (p= 0.994)	-0.01	ALAN = Night (p= 0.671)	-0.42
		ALAN = Day (p= 0.180)	1.34	ALAN ≠ Day **(p= 0.001)**	3.214
Nidopallium Caudolateral	Motor	ALAN = Night (p= 0.462)	-0.74	ALAN ≠ Night **(p= 0.011)**	-2.55
		ALAN = Day (p= 0.082)	-1.74	ALAN ≠ Day **(p= 0.005)**	-2.81
Nidopallium adjacent to the Core of the Entopallium	Visual	ALAN = Night (p= 0.716)	-0.36	ALAN ≠ Night **(p= 0.019)**	-2.34
		ALAN = Day (p= 0.682)	-0.41	ALAN = Day (p= 0.411)	0.82
Posterior Dorsal Mesopallium	Visual	ALAN = Night (p= 0.149)	-1.44	ALAN = Night (p= 0.173)	-1.36
		ALAN = Day (p= 0.153)	-1.43	ALAN = Day (p= 0.205)	-1.27
Posterior Hyperpallium	Visual	ALAN ≠ Night **(p= 0.009)**	-2.60	ALAN = Night (p= 0.079)	-1.76
		ALAN ≠ Day **(p= 0.017)**	-2.39	ALAN = Day (p= 0.072)	-1.80
Posterior Lateral Ventral Mesopallium	Motor	ALAN = Night (p= 0.339)	-0.96	ALAN = Night (p= 0.363)	-0.91
		ALAN = Day (p= 0.297)	-1.04	ALAN = Day (p= 0.203)	-1.27
Septum		ALAN = Night (p= 0.821)	-0.23	ALAN ≠ Night **(p= 0.039)**	2.06
		ALAN = Day (p= 0.976)	-0.03	ALAN = Day (p= 0.051)	1.95
Striatum adjacent to the Core of the Entopallium	Visual	ALAN ≠ Night **(p< 0.0001)**	-215.8	ALAN = Night (p= 0.608)	-0.51
		ALAN ≠ Day **(p< 0.0001)**	-207.5	ALAN = Day (p= 0.326)	0.98

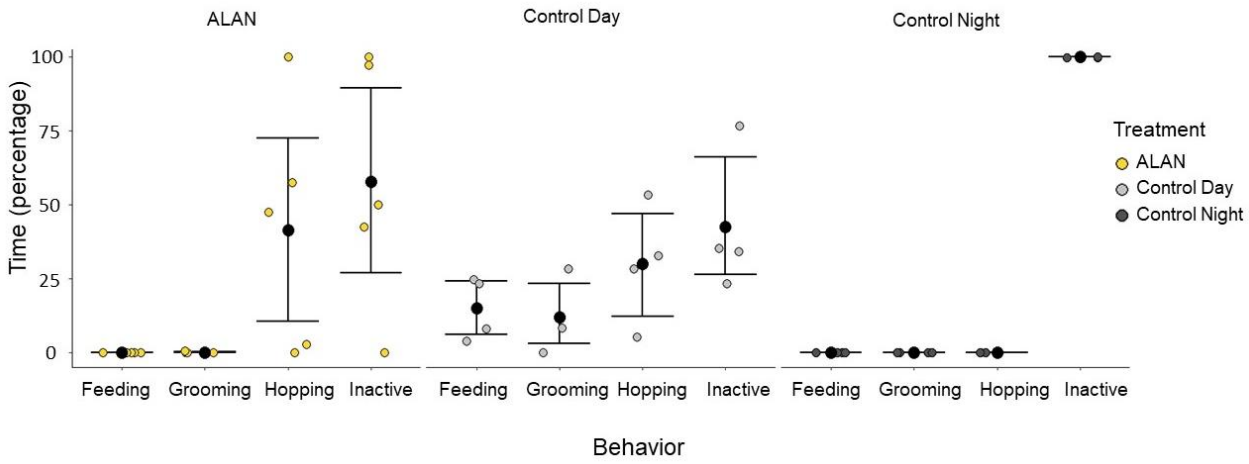


Figure 1. Types of behavior 75 to 105 minutes before perfusion for birds exposed to ALAN and control birds collected during the day and night. A 30-minute window 90 minutes before perfusion (75 to 105 minutes) was analyzed and broken down into four different behaviors, feeding (eating or drinking), grooming, hopping, and inactive. Shown are means + - 1 SE.

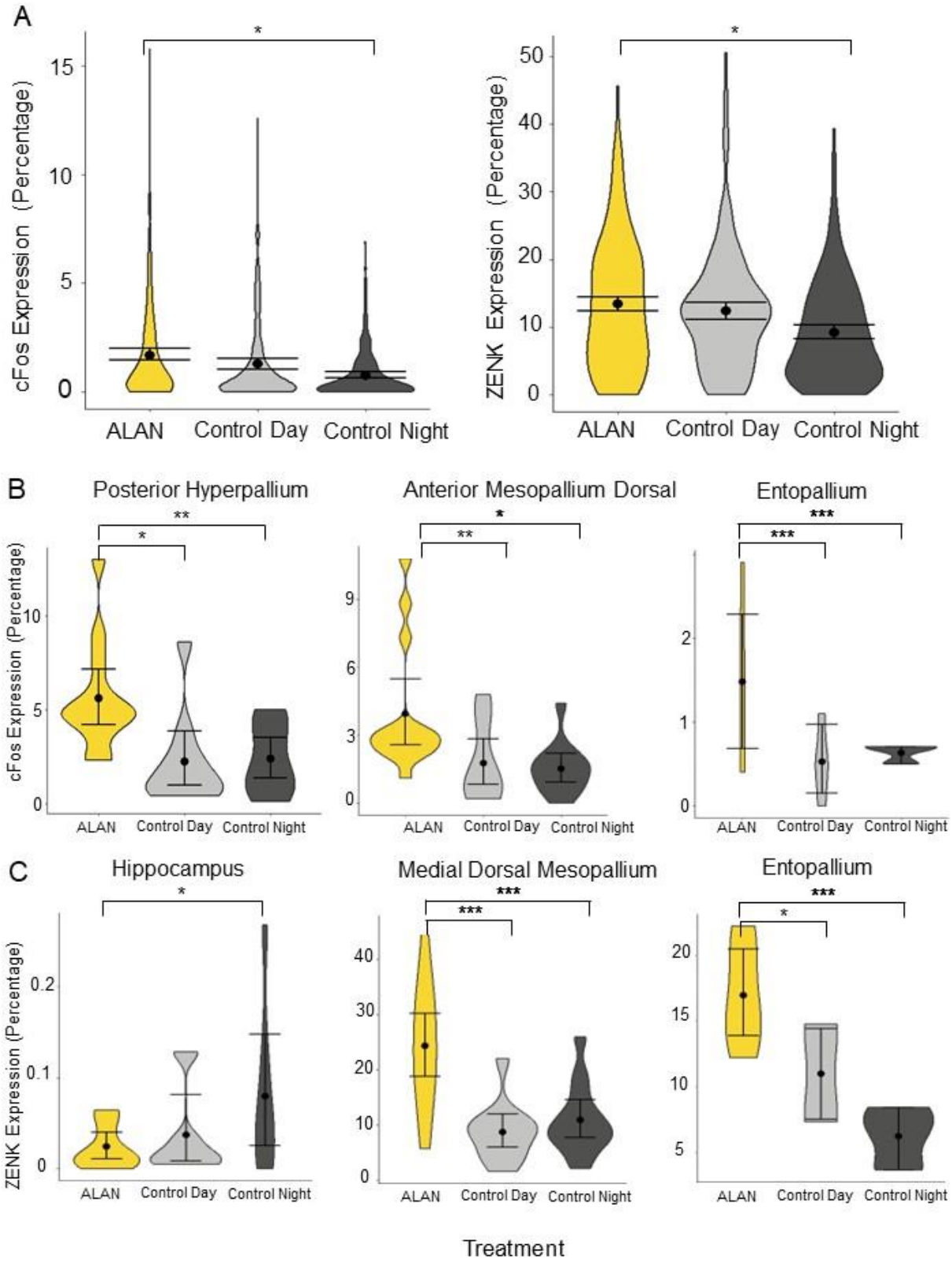
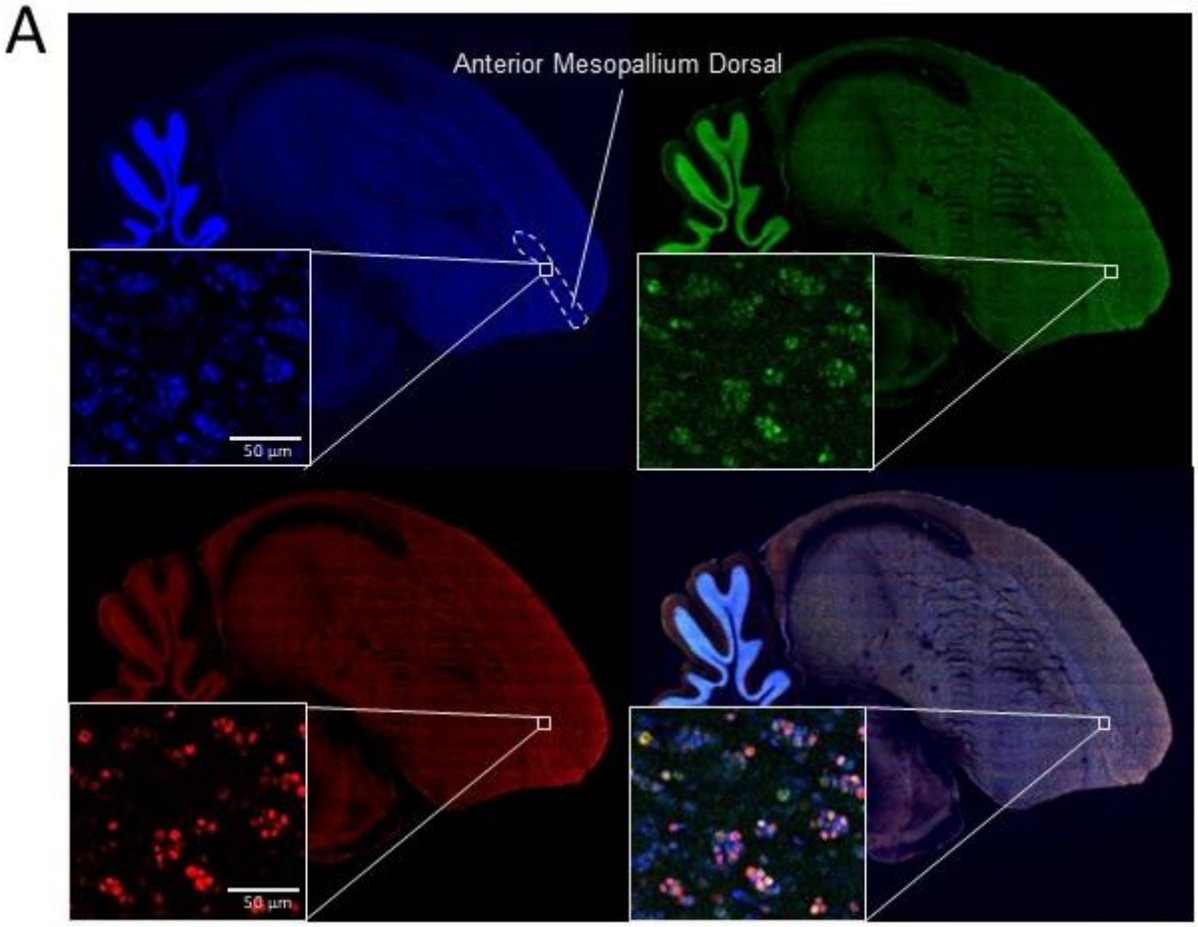

A
cFos Expression (Percentage)
ZENK Expression (Percentage)
ALAN
Control Day
Control Night
B
Posterior Hyperpallium
Anterior Mesopallium Dorsal
Entopallium
C
Hippocampus
Medial Dorsal Mesopallium
Entopallium
Treatment

Figure 2. Immediate early gene expression of cFos and ZENK throughout the brain for birds exposed to ALAN, and control birds collected during subjective day and night. (A) Total cFos and ZENK expression, shown in percentages. Expression is significantly higher in the ALAN treatment group compared to the night controls but not the day controls. (B) cFos expression (percentage) comparing birds exposed to ALAN to control day and control night groups in three brain regions: posterior hyperpallium, anterior mesopallium dorsal, and entopallium. (C) ZENK expression (percentage) comparing birds exposed to ALAN to control day and control night groups in three brain regions: hippocampus, medial dorsal mesopallium, and entopallium. Displayed are representative brain regions from a priori hypotheses, please see Figures S2 and S3 for all brain regions. Shown are means ± 1 SE. Significance stars: '*' $p < 0.05$, '**' $p < 0.01$, '***' $p < 0.001$.


A
Anterior Mesopallium Dorsal
50 µm
50 µm

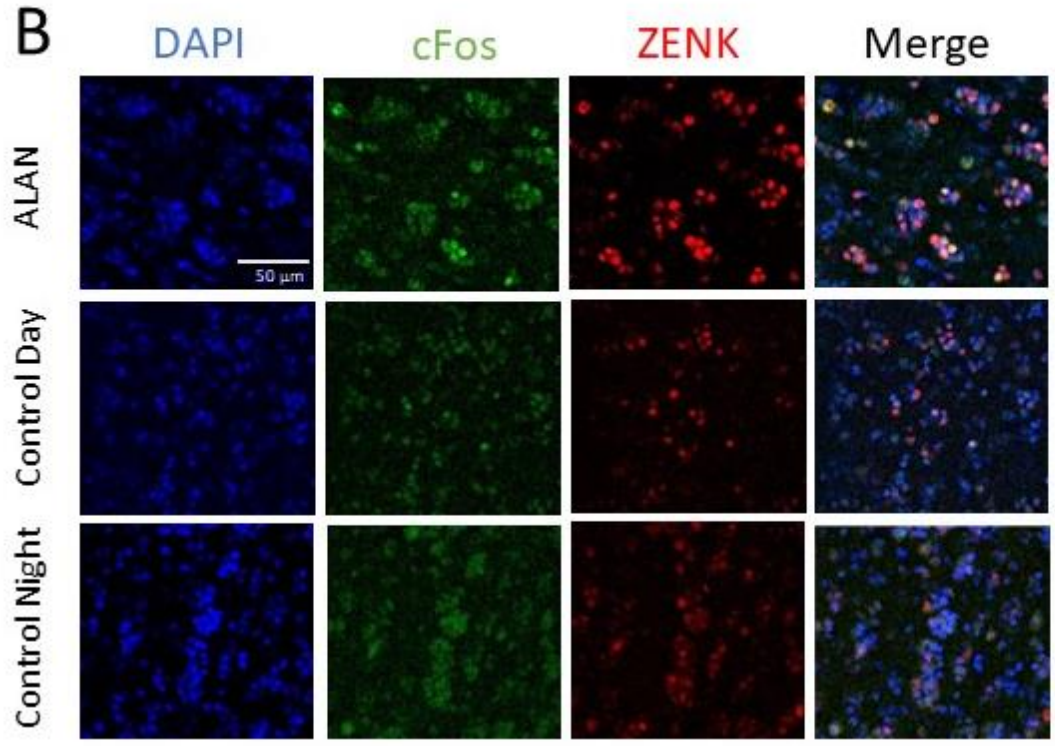

B
DAPI
cFos
ZENK
Merge
ALAN
Control Day
Control Night
50 µm

Figure 3. Brain slices with cFos and ZENK staining in the anterior mesopallium dorsal. (A) A sagittal slice of a representative zebra finch brain 1 mm from the center, showing the anterior mesopallium dorsal. Blue is DAPI, green is cFos, and red is ZENK expression. (B) Images from the anterior mesopallium dorsal of cFos, ZENK, and the overlay of both with DAPI for a bird exposed to ALAN, a bird collected during the day (control day), and a bird collected at night (control night).

References

1. D. M. Dominoni, R. J. Nelson, Artificial light at night as an environmental pollutant: An integrative approach across taxa, biological functions, and scientific disciplines. *Journal of Experimental Zoology Part a-Ecological and Integrative Physiology* **329**, 387-393 (2018).

2. J. Q. Ouyang, S. Davies, D. Dominoni, Hormonally mediated effects of artificial light at night on behavior and fitness: linking endocrine mechanisms with function. *Journal of Experimental Biology* **221**, (2018).

3. V. J. Alaasam *et al.*, Effects of dim artificial light at night on locomotor activity, cardiovascular physiology, and circadian clock genes in a diurnal songbird. *Environmental Pollution* **282**, (2021).

4. K. Spoelstra, I. Verhagen, D. Meijer, M. E. Visser, Artificial light at night shifts daily activity patterns but not the internal clock in the great tit (Parus major). *Proceedings of the Royal Society B-Biological Sciences* **285**, (2018).

5. D. M. Dominoni *et al.*, Artificial light at night leads to circadian disruption in a songbird: integrated evidence from behavioural, genomic and metabolomic data. *bioRxiv*, 2020.2012.2018.423473 (2021).

6. V. J. Alaasam *et al.*, Light at night disrupts nocturnal rest and elevates glucocorticoids at cool color temperatures. *Journal of Experimental Zoology Part a-Ecological and Integrative Physiology* **329**, 465-472 (2018).

7. I. Mishra *et al.*, Light at night disrupts diel patterns of cytokine gene expression and endocrine profiles in zebra finch (Taeniopygia guttata). *Scientific Reports* **9**, (2019).

8. L. W. Zhang *et al.*, Melatonin modulates monochromatic light-induced melatonin receptor expression in the hypothalamus of chicks. *Acta Histochemica* **119**, 733-739 (2017).

9. Y. El-Sherif, J. Tesoriero, M. V. Hogan, A. Wieraszko, Melatonin regulates neuronal plasticity in the hippocampus. *Journal of Neuroscience Research* **72**, 454-460 (2003).

10. M. A. Kus *et al.*, Effects of melatonin hormone on hippocampus in pinealectomized rats: An immunohistochemical and biochemical study. *Neuroendocrinology Letters* **34**, 418-425 (2013).

11. V. Chabot, M. Caldani, M. M. de Reviers, J. Pelletier, Localization and quantification of melatonin receptors in the diencephalon and posterior telencephalon of the sheep brain. *Journal of Pineal Research* **24**, 50-57 (1998).

12. S. Moaraf *et al.*, Artificial light at night affects brain plasticity and melatonin in birds. *Neuroscience Letters* **716**, (2020).

13. S. T. Taufique, A. Prabhat, V. Kumar, Light at night affects hippocampal and nidopallial cytoarchitecture: Implication for impairment of brain function in diurnal corvids. *Journal of Experimental Zoology Part a-Ecological and Integrative Physiology* **331**, 149-156 (2019).

14. D. Namgyal *et al.*, Dim Light at Night Induced Neurodegeneration and Ameliorative Effect of Curcumin. *Cells* **9**, (2020).

15. S. Moaraf *et al.*, Evidence That Artificial Light at Night Induces Structure-Specific Changes in Brain Plasticity in a Diurnal Bird. *Biomolecules* **11**, (2021).

16. M. E. Greenberg, A. L. Hermanowski, E. B. Ziff, EFFECT OF PROTEIN-SYNTHESIS INHIBITORS ON GROWTH-FACTOR ACTIVATION OF C-FOS, C-MYC, AND ACTIN GENE-TRANSCRIPTION. *Molecular and Cellular Biology* **6**, 1050-1057 (1986).

17. S. W. Flavell, M. E. Greenberg, Signaling mechanisms linking neuronal activity to gene expression and plasticity of the nervous system. *Annual Review of Neuroscience* **31**, 563-590 (2008).

18. J. F. Guzowski *et al.*, Mapping behaviorally relevant neural circuits with immediate-early gene expression. *Current Opinion in Neurobiology* **15**, 599-606 (2005).

19. G. Feenders *et al.*, Molecular Mapping of Movement-Associated Areas in the Avian Brain: A Motor Theory for Vocal Learning Origin. *Plos One* **3**, (2008).

20. J. I. Morgan, T. Curran, CALCIUM AS A MODULATOR OF THE IMMEDIATE-EARLY GENE CASCADE IN NEURONS. *Cell Calcium* **9**, 303-311 (1988).

21. S. M. Sagar, F. R. Sharp, T. Curran, EXPRESSION OF C-FOS PROTEIN IN BRAIN - METABOLIC MAPPING AT THE CELLULAR-LEVEL. *Science* **240**, 1328-1331 (1988).

22. K. J. O'Donovan, W. G. Tourtellotte, J. Milbrandt, J. M. Baraban, The EGR family of transcription-regulatory factors: progress at the interface of molecular and systems neuroscience. *Trends in Neurosciences* **22**, 167-173 (1999).

23. G. C. Nordmann *et al.*, A high sensitivity ZENK monoclonal antibody to map neuronal activity in Aves. *Scientific Reports* **10**, (2020).

24. Y. Nakane, T. Yoshimura, Universality and diversity in the signal transduction pathway that regulates seasonal reproduction in vertebrates. *Frontiers in Neuroscience* **8**, (2014).

25. D. Durstewitz, S. Kroner, O. Gunturkun, The dopaminergic innervation of the avian telencephalon. *Progress in Neurobiology* **59**, 161-195 (1999).

26. S. Watanabe, U. Mayer, H. J. Bischof, Visual Wulst analyses "where" and entopallium analyses "what" in the zebra finch visual system. *Behavioural Brain Research* **222**, 51-56 (2011).

27. S. Watanabe, U. Mayer, H. J. Bischof, Pattern discrimination is affected by entopallial but not by hippocampal lesions in zebra finches. *Behav Brain Res* **190**, 201-205 (2008).

28. C. C. Damphousse, N. Miller, D. F. Marrone, Dissociation of spatial and object memory in the hippocampal formation of Japanese quail. *Iscience* **25**, (2022).

29. X. G. He, L. Xiao, N. Sui, Effects of SCH23390 and spiperone administered into medial striatum and intermediate medial mesopallium on rewarding effects of morphine in day-old chicks. *European Journal of Pharmacology* **627**, 136-141 (2010).

30. U. Mayer, S. Watanabe, H. J. Bischof, Spatial memory and the avian hippocampus: Research in zebra finches. *Journal of Physiology-Paris* **107**, 2-12 (2013).

31. V. Bingman, P. Ioale, G. Casini, P. Bagnoli, The avian hippocampus: evidence for a role in the development of the homing pigeon navigational map. *Behavioral neuroscience* **104**, 906 (1990).

32. Q. Liu, Z. X. Wang, J. Cao, Y. L. Dong, Y. X. Chen, Dim Blue Light at Night Induces Spatial Memory Impairment in Mice by Hippocampal Neuroinflammation and Oxidative Stress. *Antioxidants* **11**, (2022).

33. S. K. T. Taufique, A. Prabhat, V. Kumar, Illuminated night alters hippocampal gene expressions and induces depressive-like responses in diurnal corvids. *European Journal of Neuroscience* **48**, 3005-3018 (2018).

34. J. G. Klinzing, N. Niethard, J. Born, Mechanisms of systems memory consolidation during sleep. *Nature Neuroscience* **22**, 1598-1610 (2019).

35. O. Gunturkun, The avian 'prefrontal cortex' and cognition. *Current Opinion in Neurobiology* **15**, 686-693 (2005).

36. O. Gunturkun, T. Bugnyar, Cognition without Cortex. *Trends in Cognitive Sciences* **20**, 291-303 (2016).

37. K. Peng, S. C. Steele, L. Becerra, D. Borsook, Brodmann area 10: Collating, integrating and high level processing of nociception and pain. *Progress in Neurobiology* **161**, 1-22 (2018).

38. C. Herold *et al.*, The receptor architecture of the pigeons' nidopallium caudolaterale: an avian analogue to the mammalian prefrontal cortex. *Brain Structure & Function* **216**, 239-254 (2011).

39. M. Johnston, C. Anderson, M. Colombo, Neural correlates of sample-coding and reward-coding in the delay activity of neurons in the entopallium and nidopallium caudolaterale of pigeons (Columba livia). *Behavioural Brain Research* **317**, 382-392 (2017).

40. J. R. Bumgarner, W. H. Walker, J. A. Liu, J. C. Walton, R. J. Nelson, Dim Light at Night Exposure Induces Cold Hyperalgesia and Mechanical Allodynia in Male Mice. *Neuroscience* **434**, 111-119 (2020).

41. Y. Jin *et al.*, A somatosensory cortex input to the caudal dorsolateral striatum controls comorbid anxiety in persistent pain. *Pain* **161**, 416-428 (2020).

42. A. Carreras *et al.*, ROLE OF THE SEPTUM ON LH, FSH AND PROLACTIN SECRETION IN MALE-RATS. *Brain Research Bulletin* **13**, 339-342 (1984).

43. G. Nagarajan, A. Jurkevich, S. W. Kang, W. J. Kuenzel, Anatomical and functional implications of corticotrophin-releasing hormone neurones in a septal nucleus of the avian brain: an emphasis on glial-neuronal interaction via V1a receptors in vitro. *Journal of Neuroendocrinology* **29**, (2017).

44. W. Vale, J. Spiess, C. Rivier, J. Rivier, CHARACTERIZATION OF A 41-RESIDUE OVINE HYPOTHALAMIC PEPTIDE THAT STIMULATES SECRETION OF CORTICOTROPIN AND BETA-ENDORPHIN. *Science* **213**, 1394-1397 (1981).

45. T. M. Ballard, A. J. Hunter, G. W. Bennett, Effect of a thyrotrophin-releasing hormone analogue, RX77368, an AMPA-induced septal hippocampal lesioned rats in an operant delayed non-matching to position test. *Psychopharmacology* **127**, 265-275 (1996).

46. N. Fabris, E. Mocchegiani, M. Provinciali, PITUITARY-THYROID AXIS AND IMMUNE-SYSTEM - A RECIPROCAL NEUROENDOCRINE-IMMUNE INTERACTION. *Hormone Research* **43**, 29-38 (1995).

47. C. Nyakas, E. R. D. Kloet, B. Bohus, HIPPOCAMPAL FUNCTION AND PUTATIVE CORTICOSTERONE RECEPTORS - EFFECT OF SEPTAL-LESIONS. *Neuroendocrinology* **29**, 301-312 (1979).

48. L. Y. Garces, Iturzaet.Nf, C. Richards, A. Drash, F. M. Kenny, EFFECT OF VARIOUS STRESSES ON CORTISOL SECRETION RATE (CSR). *Journal of Pediatrics* **72**, 566-+ (1968).

49. S. Moaraf *et al.*, Artificial Light at Night Increases Recruitment of New Neurons and Differentially Affects Various Brain Regions in Female Zebra Finches. *International Journal of Molecular Sciences* **21**, (2020).

Chapter 3: Birds of a feather flock together: Light pollution's effects differ by social context.

Abstract

Artificial light at night (ALAN), a growing pervasive pollutant, disrupts physiological and behavioral rhythms across organisms. Social interactions play a significant role in shaping individual and group biological rhythms, but they have often been overlooked in the context of environmental stressors such as ALAN. In this study, we explore how dim ALAN affects zebra finches (*Taeniopygia guttata*) in social and isolated environments, examining behavioral, physiological, and molecular rhythms. We found that social birds under ALAN had an earlier activity onset and greater disruption in hypothalamic and liver circadian gene expression than control or isolated counterparts under ALAN. Additionally, we found that activity onset significantly correlated negatively with hypothalamic *Bmal1* and *Cry1* expression and positively with *Per2* expression in birds exposed to ALAN. We show that social interactions may exacerbate the effects of ALAN, which highlights the critical need to consider social contexts in biological studies to better mimic natural conditions.

Introduction

The advent of artificial light at night (ALAN) presents a formidable challenge to daily life, with the potential to disrupt the delicate balance of the circadian system in molecular, physiological, and behavioral rhythms, thereby impacting overall health (*27*). Organisms across different species synchronize their biological rhythms with external cues, such as light and temperature, to maintain alignment with the natural day-night cycle. At the heart of these rhythms is the circadian clock, governed by a feedback loop of oscillating core genes. In this system, Clock (*Clk*) and Brain and muscle Arnt-like protein-1 (*Bmal1*) genes promote Period (*Per*) and Cryptochrome (*Cry*) expression, which subsequently suppress their own activity (*3*). This system is entrained to environmental cues, primarily by the degradation of the PER/CRY protein complex in light (*3*). The master clock, located in the suprachiasmatic nucleus (SCN) nestled in the hypothalamus, orchestrates peripheral clocks in other tissues, such as the liver, which can also entrain downstream physiology and behaviors, like hormone secretion and activity periods, ensuring that the organism's internal rhythms are in harmony with environmental cycles (*21*).

Melatonin, produced by the pineal gland predominantly during the night, serves as a critical signal for sleep readiness and influences various biological functions by aligning with the day-night cycle (*85*). Melatonin production is tightly controlled by the circadian clock, creating a vital link between the external

environment's light-dark cycle and the organism's internal biological processes. However, in birds, ALAN suppresses melatonin production during the night (*47, 86*).

Despite the established disruptive effects of ALAN on a wide variety of circadian regulation from neuronal activity to behavior (*40, 47, 87*), much of the existing research has concentrated on isolated animal models or housing conditions have largely been ignored (*28, 47*). Yet social interactions play a pivotal role in shaping circadian regulation and behavioral rhythms, suggesting a complex interplay between social environments and the internal biological clock (*31, 33, 88*). In social settings, organisms often synchronize their activities and physiological processes for various benefits such as enhanced reproductive success and chance of survival (*32, 89, 90*). In flies, groups align activity rhythms and, interestingly, the introduction of a short-period mutant can shorten the group's natural rhythm (*18*). Additionally, a recent study showed that paired birds can restore rhythmic activity in an arrhythmic environment of constant light (*34*). Therefore, our study aims to explore whether social conditions can alter the impacts of ALAN, potentially mitigating or exacerbating its effects on circadian regulation. This investigation will provide a deeper understanding of how social and environmental factors intertwine to influence the biological clocks of organisms exposed to unnatural light conditions.

We exposed zebra finches (*Taeniopygia guttata*), a social diurnal model species, to ALAN in both isolated and social conditions. We compared activity levels, circadian gene expression in the hypothalamus and liver, and melatonin to

birds in control dark night conditions, housed in either isolated or social settings. We chose these metrics for a comprehensive analysis of clock changes responding to ALAN, aiming to explore core and peripheral mechanistic clock changes and their interactions. If social conditions provide circadian rescue, we predicted that circadian disruption, *i.e.*, misalignment of gene-expression, physiology and behavior with light-dark conditions, would be less in ALAN-exposed birds housed socially than in isolated conditions. Alternatively, ALAN could be a strong enough *zeitgeber* or stressor that the social context has no effect. In this scenario, ALAN exposure would trigger similar disruptive responses across both social and isolated conditions. This study aims to clarify the role of social settings in modulating the effects of environmental disturbances on biological rhythms.

Methods

Experimental Design

We housed 99 zebra finches individually (n=53; 47 x 31 x 36cm cages) or grouped indoors (n=46, 47 x 93 x 36 cm cages) and entrained them to 12 hours light and 12 hours dark (12L:12D) for three weeks. Grouped (social) cages held 3 males and 3 females. For daylight, we used 1.4-Watt 5000 K light emitting diode (LED) rated at 95 Lumens lights at 9:00 (zeitgeber time (ZT) 0) and lights off at 21:00 (ZT 12). Birds were provided food and water *ad libitum*. Each cage contained a mechanized perch that relayed hop activity to MATLAB every minute. Cages had individual light-occlusion shades and constant white noise in the background to

limit visual and acoustic cues across cages. We also video-recorded cages containing groups of birds every half hour for two minutes (*11*).

We randomly assigned birds to one of four conditions: social ALAN (n=24, 12L:12L dim), isolated ALAN (n=26, 12L:12L dim), social control (n=22, 12L:12D), and isolated control (n=27, 12L:12D). ALAN was standardized to ~5 lux ± 0.01 from a 20 x 1.5 cm 5000 K broad spectrum LED strip using an Extech Easyview Digital Light Meter (model EA13) and lux was calculated using a mean measurement at perch height and two opposing base corners. For a full-spectrum description of the lights, please see (*28*). As determined by One-Way ANOVA, groups did not differ in initial mass ($p= 0.25$). After a 3-week entertainment period, we exposed individuals to ALAN or continued control conditions for 10 days. We then sacrificed the birds at four time points: ZT 1, ZT 7, ZT 13, and ZT19.

To acquire individual-based melatonin data, we repeated the experiment with new birds, collecting within-individual blood samples at four different times (ZT 1, ZT 7, ZT 13, and ZT 19). We collected blood samples after nine days of ALAN exposure at 4 different time points over 10 days (no more than 1% of their body mass per 48 hours).

Real-Time qPCR

We used real-time PCR quantification with SYBR-Green to detect circadian gene expression in the hypothalamus and liver (*28*). We homogenized the tissues and analyzed in triplicate for technical repeats. We isolated total tissue RNA using

Trizol (Life Technologies, Carlsbad, California) and quantified it using Nanodrop 1000 (Thermo Scientific). Reverse transcription was done from 3 mg of total RNA through Versco cDNA synthesis kit. We designed the primers using Primer 3 based on Zebra Finch C*ry1*, *Bmal1*, *Per2*, and *Per3* genes (Table S1). Amplicon abundance was calculated using the $2^{-\Delta\Delta CT}$ method.

Melatonin

We measured plasma melatonin concentrations using an enzyme-linked immunoassay kit (Aviva Systems Biology OKEH02566) on 96-well plates according to manufacturer procedures and validated for zebra finches (*28*). When available, 25 mL of plasma was diluted (2X) and run in duplicate. The plate was read at 450 nm using a standard microplate reader (BioTek Synergy HTX multi-mode reader) and BioteGen5 data analysis software (BioTek Instruments, Inc, Winooski, Vermont). The interplate coefficient of variation (CV) was 6.4% and the intraplate CV was 5.3%. To increase accuracy, we normalized melatonin levels within each experimental round, ran 6 months apart.

Statistical Analyses

We analyzed all data using R version 4.1.2 (R Development Core Team, 2019). A Welch two-sample t-test was used to test for differences in nocturnal activity between control and ALAN for individually caged and social birds. We used the program Chronoshop 1.1 (freely available; see supplementary) to calculate activity

onset (the first time point at which activity is higher than the average) and activity offset (the final time point at which activity is higher than the average) for each day relative to lights on and off. An ANOVA with Tukey's post hoc comparison was used to determine differences in activity on and offset. Cosinor (version 1.2.3 (Barnett and Dobson, 2010)) was used for rhythmic analysis of melatonin to test for treatment effects on amplitude (i.e. the difference between peak and the mean value of wave) and phase (i.e. time of peak expression in wave). A Student's t-test with Welch's corrections was used to test for the effects of ALAN on circadian gene expression at each timepoint. CircaCompare (version 0.1.1) was used for rhythmic analysis of gene expression. We used linear regression models to compare gene expression with activity onset. All models met assumptions and significance was taken at $\alpha=0.05$.

Ethics Statement

All procedures were conducted in accordance with the National Institute of Health Ethical Use of Animals and approved by the University of Nevada, Reno Institutional Animal Care, and Use Committee.

Results

Activity

Exposure to ALAN significantly increased nocturnal activity in zebra finches (Figure 1). Perch recordings revealed a notable rise in total nocturnal activity for both isolated ($t = 7.18$, $p < 0.01$) and social conditions ($t = 6.84$, $p < 0.01$) under ALAN. Additionally, ALAN exposure led to earlier activity onset and delayed offset times across both housing conditions, indicating an extension of the active period in response to artificial lighting (Figure 1B). Birds exposed to ALAN significantly increased their activity onset (Isolated: Diff = 65.23, $p < 0.01$, Social: Diff = 123.58, $p < 0.01$) and lengthened their offset (Isolated: Diff = -53.92, $p < 0.01$, Social: Diff = -77.97, $p < 0.01$) compared to their controls. However, social birds had a significantly earlier activity onset than isolated birds when exposed to ALAN (-66.65, $p < 0.01$) and later offset (98.92, $p < 0.01$).

Gene Expression

To determine activity onset origin, we analyzed core circadian genes in the hypothalamus and peripheral genes in the liver. We observed consistent daily rhythms in the expression of circadian genes *Cry1, Per2,* and *Per3* within the hypothalamus across all treatments (all p-values < 0.01: Figure 2; Figure S1). In contrast, *Bmal1* expression exhibited less pronounced rhythmicity, with variability observed across different conditions (Isolated control: $p = 0.08$, Isolated ALAN: $p = 0.01$, Social control: $p = 0.03$, Social ALAN: $p < 0.01$). No significant differences were found in the phase or amplitude of *Cry1, Per2,* and *Per3* expressions between control and ALAN-exposed groups (Table S2). We identified a significant phase shift

in *Bmal1* expression among social ALAN birds compared to their social controls ($p < 0.01$) and not in isolated conditions ($p = 0.77$). *Bmal1* expression significantly decreased among socially housed birds under ALAN at ZT 13 ($t = -3.30$, $p = 0.01$; Figure 2), that again was not seen in isolated birds ($t = 0.22$, $p = 0.83$). However, regardless of social condition *Per3* expression significantly decreased in birds exposed to ALAN relative to their respective controls at ZT 1 (Isolated: $t = -2.48$, $p = 0.03$, Social: $t = -2.42$, $p = 0.04$).

Robust daily oscillations were also found in *Bmal1, Cry1, Per2,* and *Per3* expression in the livers, consistent across all treatments (all p-values < 0.01: Table S2). Comparisons of phase and amplitude between control groups and those exposed to ALAN showed no significant differences for *Bmal1, Cry1,* and *Per2* expressions. However, the amplitude of *Per3* expression increased in socially housed birds exposed to ALAN ($p = 0.05$), but not isolated birds ($p = 0.26$). Differences in individual timepoints were insignificant between isolated birds exposed to ALAN and controls. However, ALAN exposure significantly decreased *Bmal1* expression at ZT 13 ($t = -2.60$, $p = 0.03$) and *Per3* at ZT 7 ($t = -2.68$, $p = 0.05$) in socially housed birds.

We conducted a correlation matrix analysis to examine the organization between gene expression levels in the hypothalamus and liver among birds subjected to ALAN exposure, either isolated or social, in the early morning (ZT 1) and early night (ZT 13) (Figure 3A) as these timepoints are the closest to activity onset and offset. We see a different organization of correlating genes between isolated

and social birds exposed to ALAN. In the early night (ZT 13), we see a stronger desynchronization of hypothalamic and liver gene expression in the socially housed birds.

Additionally, we found that hypothalamic circadian genes (*Bmal1, Cry1,* and *Per2*) expressed early in the morning (ZT 1) strongly predict (Figure 3B; all $p < 0.01$) activity onset of the last experimental day under ALAN. Control birds maintained an onset close to time 0, so it was unnecessary to include them in the analyses.

Melatonin

We measured melatonin levels to determine if the altered circadian genes were disrupting downstream physiological rhythms. Melatonin concentrations oscillated throughout the day in all groups. The amplitude (Isolated: $z = 0.12$, $p = 0.73$; Social: $z = 0.04$, $p = 0.84$) and phase (Isolated: $z = 0.06$, $p = 0.81$; Social: $z = 0.16$, $p = 0.69$) of melatonin did not differ between birds exposed to ALAN and controls regardless of social condition (Figure S2).

Discussion

We show that central and peripheral circadian gene expression were exacerbated when birds were housed together, leading to increased desynchronization of correlated brain and liver expression. Advanced activity onset was also strongly correlated to gene expression in the hypothalamus but did not

affect downstream melatonin expression. We highlight that social context strongly affects gene expression and circadian misalignment, exacerbating responses to external stressors.

ALAN disrupts circadian rhythms in both behavioral patterns and gene expression, but there has been debate in the field as to whether these two are connected (*28, 29, 41*). We found ALAN exposure increased nocturnal activity and caused earlier activity onset, which was intensified by social interactions. Furthermore, ALAN exposure decreased *Per3* expression in the hypothalamus uniformly, but *Bmal1* expression was only disrupted in social birds in central (hypothalamus) and peripheral (liver) clocks. The interaction of ALAN's effects across social conditions was supported by the relationship of circadian hypothalamic genes on activity onset. Hypothalamic expression in the early morning of *bmal1*, *cry1*, and *per2* significantly predicted activity onset. In social groups, these genes' expression was more severely affected by ALAN, correlating with greater alterations in activity patterns. This supports the argument for an association between behavioral and core clock shifts due to ALAN.

The central clock in the SCN performs the crucial role of synchronizing peripheral clocks across an organism, with desynchronization leading to health deficits (*4, 91*). ALAN, and other stressors, have the capability of disrupting peripheral rhythms even if behavioral rhythms or the core clock are untouched, desynchronizing the organism's system (*92, 93*). We found that ALAN reorganized the relationship of circadian genes differently in birds that were isolated compared

to grouped. Different patterns emerged between the two treatments and in the early night we saw increased desynchronization of central and peripheral clocks particularly for social birds. Therefore, in social settings, ALAN not only disrupts both core and peripheral clocks but also leads to a greater degree of desynchronization in their relationship compared to isolated birds.

Contrary to our hypothesis, we observed no significant differences in melatonin levels across treatment groups, suggesting that the mechanism by which ALAN and social interactions affect circadian rhythms may not directly involve melatonin suppression. There are mixed findings on ALAN's ability to suppress melatonin (*28, 42, 47, 94*). Some prior studies reported ALAN-induced melatonin disruption in birds, housed individually or socially (*42, 47, 95*). However, our lab previously found no suppression of melatonin in bird housed individually (*28*) and another study in humans found negative effects from ALAN independent of melatonin excretion (*94*). These discrepancies may be due to species, light intensity, and individual variation, demonstrating the complex interplay of physiological responses to environmental change.

The synchronization of circadian rhythms, facilitated by social cues, is a critical aspect of social coordination. However, within flies, an individual with a disrupted timing can propagate this disruption throughout an entire group, allowing them to adopt the altered rhythm (*18*). This mechanism may explain why in our study social birds under ALAN showed greater disruption in circadian gene expression, desynchronization, and activity patterns compared to their isolated

counterparts. We speculate that individuals within a social group who are particularly sensitive to ALAN might influence the circadian rhythm of the entire group.

Our study's results are the first of our knowledge to demonstrate a molecular shift in circadian regulation due to social interactions in vertebrates. This study emphasizes the role of social context in understanding the effects of environmental disturbances like ALAN. The exacerbated responses observed in social settings suggest that collective behaviors might amplify responses to light pollution. This has significant implications for understanding the ecological impacts of ALAN, as social dynamics and group living are common across many taxa. Furthermore, these findings highlight the importance of incorporating social contexts into research designs to obtain more relevant insights into the biological impacts of environmental stressors.

In conclusion, our investigation into the effects of ALAN on zebra finches reveals that social interactions significantly amplify circadian disruptions. These findings contribute to a growing body of evidence indicating the profound impact of light pollution on biological rhythms and highlight the need for further research into mitigating these effects. Future studies should explore the underlying mechanisms of social amplification of ALAN effects and assess the ecological consequences of disrupted circadian rhythms in group-living species. The findings of this study shed light on the complex interplay between social conditions in response to ALAN

exposure and the importance of accounting for social context in experimental lab settings as results may otherwise be less applicable to natural life.

Author contributions

CH and JQO designed the experiments. CH conducted the experiments and completed data analysis. JQO and YZ oversaw the project, provided training, reviewed analyses, and provided funding. All authors contributed to and reviewed the writing.

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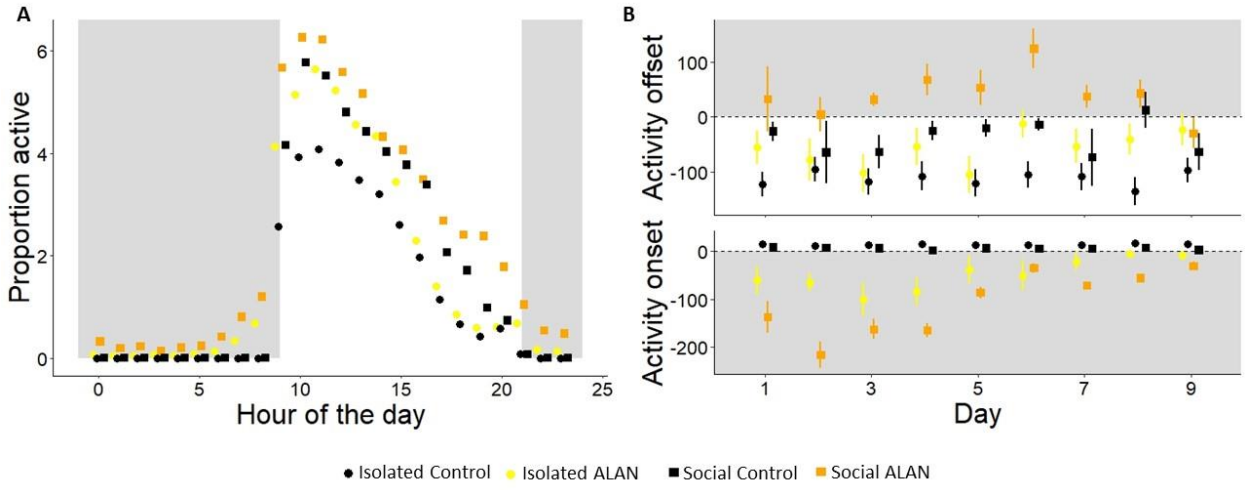

Figure 1. Activity cycles for birds exposed to dim ALAN across social conditions. (A) The mean daily activity profile over nine days, comparing ALAN-exposed birds to controls under dark night conditions. (B) Comparisons of activity onset and offset times between isolated and social conditions under ALAN exposure and control settings. Data are reported as mean ± SE.

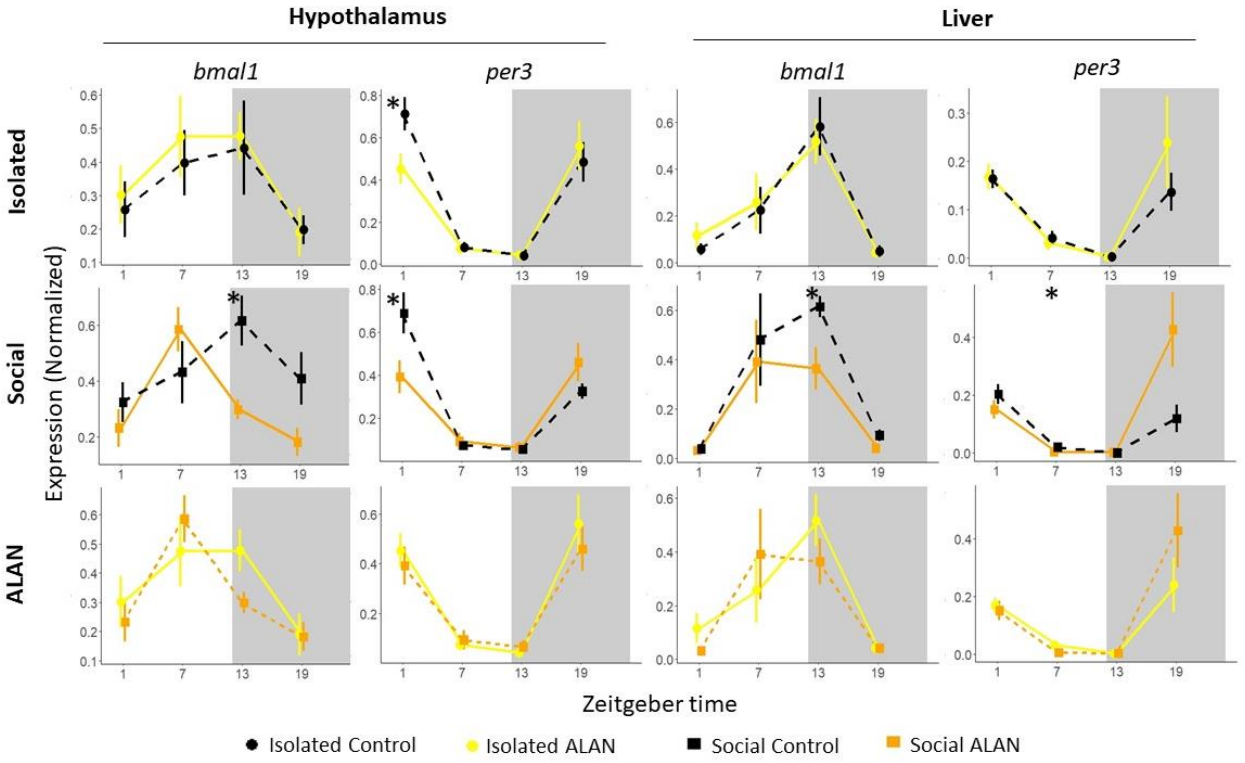

Figure 2. Daily circadian gene expression in the hypothalamus and liver under ALAN. Normalized expression of *Bmal1* and *Per3* collected at four timepoints throughout the day. Shaded portions represent nighttime (ZT 12-ZT 24). Birds

exposed to ALAN were significantly different from controls in *Per3* expression at ZT 1 (Isolated: p = 0.03, Social: p = 0.04) and only social ALAN birds were significantly different from social controls in *Bmal1* expression at ZT 13 (p = 0.01) in the hypothalamus. Birds exposed to ALAN in social conditions significantly differed from social controls in *Bmal1* expression at ZT 13 (p = 0.03) and *Per3* expression at ZT 7 (p = 0.05) in the liver. Asterisks: '*' p < 0.05, '**' p < 0.01, '***' p < 0.001.

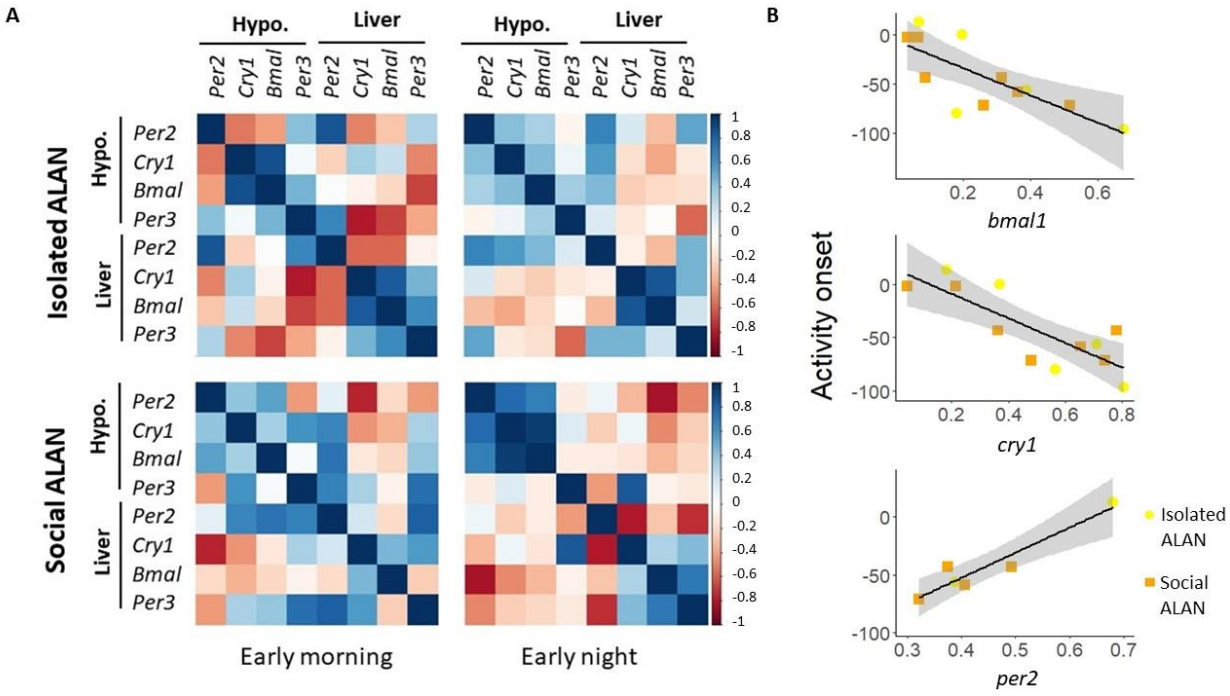

Figure 3. Correlation matrix of circadian genes expressed in the hypothalamus and liver under ALAN and prediction of activity onset. (A) Matrixes are separated by isolated or socially housed birds and time (early morning is ZT 1 and early night is 13). Dark blue shows a strong positive correlation and dark red shows a strong negative correlation between four circadian genes (*Bmal1, Cry1, Per2,* and *Per3*) in the hypothalamus and liver. (B) Circadian genes *Bmal1* (p < 0.01), C*ry1* (p < 0.01), and P*er2* (p < 0.01) expressed in the hypothalamus at ZT 1 predict activity onset of the last experimental day in birds exposed to ALAN. Lines are fitted with statistically significant linear regression models and shaded portions represent 95% confidence interval. Points represent individuals.

References

1. U. Albrecht, H. Oster, The circadian clock and behavior. *Behavioural Brain Research* **125**, 89-91 (2001).
2. M. J. Zylka, L. P. Shearman, D. R. Weaver, S. M. Reppert, Three period homologs in mammals: Differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. *Neuron* **20**, 1103-1110 (1998).
3. J. C. Dunlap, Molecular bases for circadian clocks. *Cell* **96**, 271-290 (1999).
4. A. Patke, M. W. Young, S. Axelrod, Molecular mechanisms and physiological importance of circadian rhythms. *Nature Reviews Molecular Cell Biology* **21**, 67-84 (2020).
5. R. M. Lunn *et al.*, Health consequences of electric lighting practices in the modern world: A report on the National Toxicology Program's workshop on shift work at night, artificial light at night, and circadian disruption. *Science of the Total Environment* **607**, 1073-1084 (2017).
6. M. Schilperoort, P. C. N. Rensen, S. Kooijman, Time for Novel Strategies to Mitigate Cardiometabolic Risk in Shift Workers. *Trends in Endocrinology and Metabolism* **31**, 952-964 (2020).
7. C. C. M. Kyba *et al.*, Artificially lit surface of Earth at night increasing in radiance and extent. *Science Advances* **3**, (2017).
8. A. Deprato *et al.*, The Influence of Artificial Light at Night on Asthma and Allergy, Mental Health, and Cancer Outcomes: A Systematic Scoping Review Protocol. *International Journal of Environmental Research and Public Health* **19**, (2022).
9. D. B. Boivin, P. Boudreau, Impacts of shift work on sleep and circadian rhythms. *Pathologie Biologie* **62**, 292-301 (2014).
10. D. B. Boivin, P. Boudreau, A. Kosmadopoulos, Disturbance of the Circadian System in Shift Work and Its Health Impact. *Journal of Biological Rhythms* **37**, 3-28 (2022).
11. V. J. Alaasam *et al.*, Light at night disrupts nocturnal rest and elevates glucocorticoids at cool color temperatures. *Journal of Experimental Zoology Part a-Ecological and Integrative Physiology* **329**, 465-472 (2018).
12. J. Q. Ouyang *et al.*, Restless roosts: Light pollution affects behavior, sleep, and physiology in a free-living songbird. *Global Change Biology* **23**, 4987-4994 (2017).
13. D. M. Dominoni *et al.*, Integrated molecular and behavioural data reveal deep circadian disruption in response to artificial light at night in male Great tits (Parus major). *Scientific Reports* **12**, (2022).
14. C. Dubowy, A. Sehgal, Circadian Rhythms and Sleep in Drosophila melanogaster. *Genetics* **205**, 1373-1397 (2017).
15. M. W. Young, K. WagerSmith, L. Vosshall, L. Saez, M. P. Myers, Molecular anatomy of a light-sensitive circadian pacemaker in Drosophila. *Cold Spring Harbor Symposia on Quantitative Biology* **61**, 279-284 (1996).
16. M. N. Nitabach, P. H. Taghert, Organization of the Drosophila circadian control circuit. *Current Biology* **18**, R84-R93 (2008).
17. I. Edery, L. J. Zwiebel, M. E. Dembinska, M. Rosbash, TEMPORAL PHOSPHORYLATION OF THE DROSOPHILA PERIOD PROTEIN. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 2260-2264 (1994).

18. J. D. Levine, P. Funes, H. B. Dowse, J. C. Hall, Resetting the circadian clock by social experience in Drosophila melanogaster. *Science* **298**, 2010-2012 (2002).
19. S. A. Cyran *et al.*, vrille, Pdp1, and dClock form a second feedback loop in the Drosophila circadian clock. *Cell* **112**, 329-341 (2003).
20. S. R. Lone, V. K. Sharma, Social synchronization of circadian locomotor activity rhythm in the fruit fly Drosophila melanogaster. *Journal of Experimental Biology* **214**, 3742-3750 (2011).
21. V. M. Cassone, Avian circadian organization: A chorus of clocks. *Frontiers in Neuroendocrinology* **35**, 76-88 (2014).
22. A. Dawson, V. M. King, G. E. Bentley, G. F. Ball, Photoperiodic control of seasonality in birds. *Journal of Biological Rhythms* **16**, 365-380 (2001).
23. G. Wang, C. E. Harpole, A. K. Trivedi, V. M. Cassone, Circadian Regulation of Bird Song, Call, and Locomotor Behavior by Pineal Melatonin in the Zebra Finch. *Journal of Biological Rhythms* **27**, 145-155 (2012).
24. K. Roxstrom-Lindquist, I. Faye, The Drosophila gene Yippee reveals a novel family of putative zinc binding proteins highly conserved among eukaryotes. *Insect Molecular Biology* **10**, 77-86 (2001).
25. Y. Sun *et al.*, Gene expression patterns in response to pathogen challenge and interaction with hemolin suggest that the Yippee protein of Antheraea pernyi is involved in the innate immune response. *J Invertebr Pathol* **138**, 10-17 (2016).
26. J. B. Duffy, GAL4 system in Drosophila: A fly geneticist's Swiss army knife. *Genesis* **34**, 1-15 (2002).
27. D. M. Dominoni, R. J. Nelson, Artificial light at night as an environmental pollutant: An integrative approach across taxa, biological functions, and scientific disciplines. *Journal of Experimental Zoology Part a-Ecological and Integrative Physiology* **329**, 387-393 (2018).
28. V. J. Alaasam *et al.*, Effects of dim artificial light at night on locomotor activity, cardiovascular physiology, and circadian clock genes in a diurnal songbird. *Environmental Pollution* **282**, (2021).
29. K. Spoelstra, I. Verhagen, D. Meijer, M. E. Visser, Artificial light at night shifts daily activity patterns but not the internal clock in the great tit (Parus major). *Proceedings of the Royal Society B-Biological Sciences* **285**, (2018).
30. S. K. T. Taufique, A. Prabhat, V. Kumar, Constant light environment suppresses maturation and reduces complexity of new born neuron processes in the hippocampus and caudal nidopallium of a diurnal corvid: Implication for impairment of the learning and cognitive performance. *Neurobiology of Learning and Memory* **147**, 120-127 (2018).
31. G. Marimuthu, S. Rajan, M. K. Chandrashekaran, SOCIAL ENTRAINMENT OF THE CIRCADIAN-RHYTHM IN THE FLIGHT ACTIVITY OF THE MICROCHIROPTERAN BAT HIPPOSIDEROS-SPEORIS. *Behavioral Ecology and Sociobiology* **8**, 147-150 (1981).
32. N. Goel, T. M. Lee, Social cues accelerate reentrainment of circadian rhythms in diurnal female Octodon degus (Rodentia-Octodontidae). *Chronobiology International* **12**, 311-323 (1995).
33. O. Siehler, S. Wang, G. Bloch, Social synchronization of circadian rhythms with a focus on honeybees. *Philosophical Transactions of the Royal Society B-Biological Sciences* **376**, (2021).

34. N. A. Jha, V. Kumar, Female conspecifics restore rhythmic singing behaviour in arrhythmic male zebra finches. *Journal of Biosciences* **42**, 139-147 (2017).
35. S. A. Brown, G. Zumbrunn, F. Fleury-Olela, N. Preitner, U. Schibler, Rhythms of mammalian body temperature can sustain peripheral circadian clocks. *Current Biology* **12**, 1574-1583 (2002).
36. S. Sukumaran, R. R. Almon, D. C. DuBois, W. J. Jusko, Circadian rhythms in gene expression: Relationship to physiology, disease, drug disposition and drug action. *Advanced Drug Delivery Reviews* **62**, 904-917 (2010).
37. A. Silva, M. J. dos Santos, D. L. G. Gitai, J. Coelho, T. G. de Andrade, Depression and anxiety symptoms correlate with diurnal preference, sleep habits, and Per3 VNTR polymorphism (rs57875989) in a non-clinical sample. *Journal of Affective Disorders* **277**, 260-270 (2020).
38. K. C. G. Van Dycke *et al.*, Chronically Alternating Light Cycles Increase Breast Cancer Risk in Mice. *Current Biology* **25**, 1932-1937 (2015).
39. T. J. Collins, ImageJ for microscopy. *Biotechniques* **43**, 25-+ (2007).
40. J. Q. Ouyang, S. Davies, D. Dominoni, Hormonally mediated effects of artificial light at night on behavior and fitness: linking endocrine mechanisms with function. *Journal of Experimental Biology* **221**, (2018).
41. D. M. Dominoni *et al.*, Artificial light at night leads to circadian disruption in a songbird: integrated evidence from behavioural, genomic and metabolomic data. *bioRxiv*, 2020.2012.2018.423473 (2021).
42. I. Mishra *et al.*, Light at night disrupts diel patterns of cytokine gene expression and endocrine profiles in zebra finch (Taeniopygia guttata). *Scientific Reports* **9**, (2019).
43. L. W. Zhang *et al.*, Melatonin modulates monochromatic light-induced melatonin receptor expression in the hypothalamus of chicks. *Acta Histochemica* **119**, 733-739 (2017).
44. Y. El-Sherif, J. Tesoriero, M. V. Hogan, A. Wieraszko, Melatonin regulates neuronal plasticity in the hippocampus. *Journal of Neuroscience Research* **72**, 454-460 (2003).
45. M. A. Kus *et al.*, Effects of melatonin hormone on hippocampus in pinealectomized rats: An immunohistochemical and biochemical study. *Neuroendocrinology Letters* **34**, 418-425 (2013).
46. V. Chabot, M. Caldani, M. M. de Reviers, J. Pelletier, Localization and quantification of melatonin receptors in the diencephalon and posterior telencephalon of the sheep brain. *Journal of Pineal Research* **24**, 50-57 (1998).
47. S. Moaraf *et al.*, Artificial light at night affects brain plasticity and melatonin in birds. *Neuroscience Letters* **716**, (2020).
48. S. T. Taufique, A. Prabhat, V. Kumar, Light at night affects hippocampal and nidopallial cytoarchitecture: Implication for impairment of brain function in diurnal corvids. *Journal of Experimental Zoology Part a-Ecological and Integrative Physiology* **331**, 149-156 (2019).
49. D. Namgyal *et al.*, Dim Light at Night Induced Neurodegeneration and Ameliorative Effect of Curcumin. *Cells* **9**, (2020).
50. S. Moaraf *et al.*, Evidence That Artificial Light at Night Induces Structure-Specific Changes in Brain Plasticity in a Diurnal Bird. *Biomolecules* **11**, (2021).

51. M. E. Greenberg, A. L. Hermanowski, E. B. Ziff, EFFECT OF PROTEIN-SYNTHESIS INHIBITORS ON GROWTH-FACTOR ACTIVATION OF C-FOS, C-MYC, AND ACTIN GENE-TRANSCRIPTION. *Molecular and Cellular Biology* **6**, 1050-1057 (1986).
52. S. W. Flavell, M. E. Greenberg, Signaling mechanisms linking neuronal activity to gene expression and plasticity of the nervous system. *Annual Review of Neuroscience* **31**, 563-590 (2008).
53. J. F. Guzowski *et al.*, Mapping behaviorally relevant neural circuits with immediate-early gene expression. *Current Opinion in Neurobiology* **15**, 599-606 (2005).
54. G. Feenders *et al.*, Molecular Mapping of Movement-Associated Areas in the Avian Brain: A Motor Theory for Vocal Learning Origin. *Plos One* **3**, (2008).
55. J. I. Morgan, T. Curran, CALCIUM AS A MODULATOR OF THE IMMEDIATE-EARLY GENE CASCADE IN NEURONS. *Cell Calcium* **9**, 303-311 (1988).
56. S. M. Sagar, F. R. Sharp, T. Curran, EXPRESSION OF C-FOS PROTEIN IN BRAIN - METABOLIC MAPPING AT THE CELLULAR-LEVEL. *Science* **240**, 1328-1331 (1988).
57. K. J. O'Donovan, W. G. Tourtellotte, J. Milbrandt, J. M. Baraban, The EGR family of transcription-regulatory factors: progress at the interface of molecular and systems neuroscience. *Trends in Neurosciences* **22**, 167-173 (1999).
58. G. C. Nordmann *et al.*, A high sensitivity ZENK monoclonal antibody to map neuronal activity in Aves. *Scientific Reports* **10**, (2020).
59. Y. Nakane, T. Yoshimura, Universality and diversity in the signal transduction pathway that regulates seasonal reproduction in vertebrates. *Frontiers in Neuroscience* **8**, (2014).
60. D. Durstewitz, S. Kroner, O. Gunturkun, The dopaminergic innervation of the avian telencephalon. *Progress in Neurobiology* **59**, 161-195 (1999).
61. S. Watanabe, U. Mayer, H. J. Bischof, Visual Wulst analyses "where" and entopallium analyses "what" in the zebra finch visual system. *Behavioural Brain Research* **222**, 51-56 (2011).
62. S. Watanabe, U. Mayer, H. J. Bischof, Pattern discrimination is affected by entopallial but not by hippocampal lesions in zebra finches. *Behav Brain Res* **190**, 201-205 (2008).
63. C. C. Damphousse, N. Miller, D. F. Marrone, Dissociation of spatial and object memory in the hippocampal formation of Japanese quail. *Iscience* **25**, (2022).
64. X. G. He, L. Xiao, N. Sui, Effects of SCH23390 and spiperone administered into medial striatum and intermediate medial mesopallium on rewarding effects of morphine in day-old chicks. *European Journal of Pharmacology* **627**, 136-141 (2010).
65. U. Mayer, S. Watanabe, H. J. Bischof, Spatial memory and the avian hippocampus: Research in zebra finches. *Journal of Physiology-Paris* **107**, 2-12 (2013).
66. V. Bingman, P. Ioale, G. Casini, P. Bagnoli, The avian hippocampus: evidence for a role in the development of the homing pigeon navigational map. *Behavioral neuroscience* **104**, 906 (1990).
67. Q. Liu, Z. X. Wang, J. Cao, Y. L. Dong, Y. X. Chen, Dim Blue Light at Night Induces Spatial Memory Impairment in Mice by Hippocampal Neuroinflammation and Oxidative Stress. *Antioxidants* **11**, (2022).

68. S. K. T. Taufique, A. Prabhat, V. Kumar, Illuminated night alters hippocampal gene expressions and induces depressive-like responses in diurnal corvids. *European Journal of Neuroscience* **48**, 3005-3018 (2018).
69. J. G. Klinzing, N. Niethard, J. Born, Mechanisms of systems memory consolidation during sleep. *Nature Neuroscience* **22**, 1598-1610 (2019).
70. O. Gunturkun, The avian 'prefrontal cortex' and cognition. *Current Opinion in Neurobiology* **15**, 686-693 (2005).
71. O. Gunturkun, T. Bugnyar, Cognition without Cortex. *Trends in Cognitive Sciences* **20**, 291-303 (2016).
72. K. Peng, S. C. Steele, L. Becerra, D. Borsook, Brodmann area 10: Collating, integrating and high level processing of nociception and pain. *Progress in Neurobiology* **161**, 1-22 (2018).
73. C. Herold *et al.*, The receptor architecture of the pigeons' nidopallium caudolaterale: an avian analogue to the mammalian prefrontal cortex. *Brain Structure & Function* **216**, 239-254 (2011).
74. M. Johnston, C. Anderson, M. Colombo, Neural correlates of sample-coding and reward-coding in the delay activity of neurons in the entopallium and nidopallium caudolaterale of pigeons (Columba livia). *Behavioural Brain Research* **317**, 382-392 (2017).
75. J. R. Bumgarner, W. H. Walker, J. A. Liu, J. C. Walton, R. J. Nelson, Dim Light at Night Exposure Induces Cold Hyperalgesia and Mechanical Allodynia in Male Mice. *Neuroscience* **434**, 111-119 (2020).
76. Y. Jin *et al.*, A somatosensory cortex input to the caudal dorsolateral striatum controls comorbid anxiety in persistent pain. *Pain* **161**, 416-428 (2020).
77. A. Carreras *et al.*, ROLE OF THE SEPTUM ON LH, FSH AND PROLACTIN SECRETION IN MALE-RATS. *Brain Research Bulletin* **13**, 339-342 (1984).
78. G. Nagarajan, A. Jurkevich, S. W. Kang, W. J. Kuenzel, Anatomical and functional implications of corticotrophin-releasing hormone neurones in a septal nucleus of the avian brain: an emphasis on glial-neuronal interaction via V1a receptors in vitro. *Journal of Neuroendocrinology* **29**, (2017).
79. W. Vale, J. Spiess, C. Rivier, J. Rivier, CHARACTERIZATION OF A 41-RESIDUE OVINE HYPOTHALAMIC PEPTIDE THAT STIMULATES SECRETION OF CORTICOTROPIN AND BETA-ENDORPHIN. *Science* **213**, 1394-1397 (1981).
80. T. M. Ballard, A. J. Hunter, G. W. Bennett, Effect of a thyrotrophin-releasing hormone analogue, RX77368, an AMPA-induced septal hippocampal lesioned rats in an operant delayed non-matching to position test. *Psychopharmacology* **127**, 265-275 (1996).
81. N. Fabris, E. Mocchegiani, M. Provinciali, PITUITARY-THYROID AXIS AND IMMUNE-SYSTEM - A RECIPROCAL NEUROENDOCRINE-IMMUNE INTERACTION. *Hormone Research* **43**, 29-38 (1995).
82. C. Nyakas, E. R. D. Kloet, B. Bohus, HIPPOCAMPAL FUNCTION AND PUTATIVE CORTICOSTERONE RECEPTORS - EFFECT OF SEPTAL-LESIONS. *Neuroendocrinology* **29**, 301-312 (1979).
83. L. Y. Garces, Iturzaet.Nf, C. Richards, A. Drash, F. M. Kenny, EFFECT OF VARIOUS STRESSES ON CORTISOL SECRETION RATE (CSR). *Journal of Pediatrics* **72**, 566-+ (1968).

84. S. Moaraf *et al.*, Artificial Light at Night Increases Recruitment of New Neurons and Differentially Affects Various Brain Regions in Female Zebra Finches. *International Journal of Molecular Sciences* **21**, (2020).
85. N. Murakami, T. Kawano, K. Nakahara, T. Nasu, K. Shiota, Effect of melatonin on circadian rhythm, locomotor activity and body temperature in the intact house sparrow, Japanese quail and owl. *Brain Research* **889**, 220-224 (2001).
86. Y. Touitou, A. Reinberg, D. Touitou, Association between light at night, melatonin secretion, sleep deprivation, and the internal clock: Health impacts and mechanisms of circadian disruption. *Life Sciences* **173**, 94-106 (2017).
87. C. K. Hui *et al.*, Dim artificial light at night alters immediate early gene expression throughout the avian brain. *Frontiers in Neuroscience* **17**, (2023).
88. A. Rath, M. Benita, J. Doron, I. Scharf, D. Gottlieb, Social communication activates the circadian gene Tctimeless in Tribolium castaneum. *Scientific Reports* **11**, (2021).
89. K. P. Maruska, R. D. Fernald, Social Regulation of Gene Expression in the Hypothalamic-Pituitary-Gonadal Axis. *Physiology* **26**, 412-423 (2011).
90. K. Chaturvedi, A. Srivastava, S. Malik, S. Rani, The presence/absence of conspecifics modulates the circadian locomotor activity and body mass in spotted munia (Lonchura punctulata). *Chronobiology International* **41**, 105-126 (2024).
91. F. Catalano *et al.*, Circadian Clock Desynchronization and Insulin Resistance. *International Journal of Environmental Research and Public Health* **20**, (2023).
92. Y. Tahara *et al.*, Entrainment of the mouse circadian clock by sub-acute physical and psychological stress. *Scientific Reports* **5**, (2015).
93. J. C. Borniger, S. K. Maurya, M. Periasamy, R. J. Nelson, Acute dim light at night increases body mass, alters metabolism, and shifts core body temperature circadian rhythms. *Chronobiology International* **31**, 917-925 (2014).
94. K. Obayashi, K. Saeki, J. Iwamoto, Y. Ikada, N. Kurumatani, Association between light exposure at night and nighttime blood pressure in the elderly independent of nocturnal urinary melatonin excretion. *Chronobiology International* **31**, 779-786 (2014).
95. D. M. Dominoni, W. Goymann, B. Helm, J. Partecke, Urban-like night illumination reduces melatonin release in European blackbirds (Turdus merula): implications of city life for biological time-keeping of songbirds. *Frontiers in Zoology* **10**, (2013).

Conclusions

In the context of rapid technological advancements and urban expansion, the disruption of circadian rhythms presents a complex challenge to both human health and ecological balance. Circadian rhythms, our intrinsic biological clocks orchestrating physiological and behavioral processes across species, face significant threats from light pollution, a burgeoning global pollutant. This dissertation delves into the molecular, neuronal, and behavioral impacts of circadian rhythm disruption, utilizing *Drosophila melanogaster* (fruit flies) and *Taeniopygia guttata* (zebra finches) to explore the adaptive and maladaptive responses to circadian disruption. It highlights the critical roles of circadian genes in synchronizing biological functions with environmental light cycles and examines the pervasive effects of ALAN across various levels of biological organization. The dissertation comprises three chapters, each focusing on a different aspect of circadian rhythm disruption.

In the first chapter, I examined the role of the YIPPEE protein in circadian regulation within *Drosophila*, suggesting potential links between circadian systems and immune-related processes. Down-regulating YIPPEE expression extended circadian activity periods and elevated levels of essential clock genes. I discovered that lowering YIPPEE expression in circadian neurons leads to higher PER protein levels and a longer locomotor period in constant darkness, alongside increased transcription of *Clk, per*, and *tim* mRNA expression. This demonstrates YIPPEE's role

in gene modulation, potentially enhancing *Clk* transcription, which then promotes *per* and *tim* transcription. Given YIPPEE's conservation from insects to mammals and its essential role in development and immune gene expression, this protein emerges as a significant regulator in both circadian rhythm and immune function. The findings open new research avenues into YIPPEE's functions across species, with potential implications for understanding human circadian systems. This study posits YIPPEE as a key player in circadian regulation, suggesting a link between circadian rhythms and immune regulation. Further exploration should focus on unravelling the precise mechanisms at play and links to the immune system.

In the second chapter, I used zebra finches to examine the neuronal response to ALAN in birds using the expression of IEGs as a proxy for neuronal activation across 24 brain regions. I found that ALAN exposure led to brain activation patterns more similar to daytime conditions, with notable differences in six specific brain regions involved in vision, movement, learning, memory, pain processing, and hormone regulation. Among these, the entopallium, crucial for visual pattern recognition, showed significant differential activation, indicating a distinct response to ALAN as a novel visual stimulus. In terms of movement, areas associated with motor pathways exhibited varied responses, suggesting ALAN may influence motor control. ALAN exposure affected areas tied to learning and memory, with increased IEG expression in regions associated with spatial recognition and associative learning, but decreased expression in the hippocampus, a key site for memory

consolidation during the night. This suggests ALAN could impair learning and memory by dampening nocturnal hippocampal activity. Overall, the study highlights that ALAN exposure creates a unique brain state, differing significantly from both day and night conditions, affecting various brain functions. This comprehensive examination of brain activity in response to ALAN offers insights into its complex effects. Further research should focus on ALAN's long-term impacts on brain function and behavior. Although, I determined areas of the brain responding to initial ALAN exposure, research should delve into the areas responding after longer ALAN exposure and their behavioral consequences.

The final chapter explores the mitigating or exacerbating effects of social conditions on ALAN-induced disruptions, providing insights into the complex interplay between social environments and circadian regulation. I showed that social interactions among birds exacerbate the effects of ALAN on circadian rhythm disruptions, including increased desynchronization between the brain and liver timing, and advanced activity onset without affecting melatonin levels. ALAN disrupts activity patterns intensified by social contexts. In social groups, central and peripheral gene expressions, particularly for *Bmal1*, were more disrupted compared to isolated birds, associated with advanced activity onset and desynchronization between core and peripheral clocks. Despite these findings, melatonin levels remained unchanged across treatments, suggesting the mechanism of circadian disruption by ALAN may not directly involve melatonin suppression. This highlights

the complex physiological responses to ALAN, potentially varying by species, light intensity, and individual differences. I demonstrated the critical role of social context in circadian rhythm regulation, theorizing that individuals sensitive to ALAN within a group could influence the entire group's rhythm. I presented a novel insight that social interactions can induce molecular shifts in circadian regulation in vertebrates. These findings have significant implications for understanding the ecological impacts of ALAN across taxa and stress the importance of incorporating social contexts in research designs to gain a fuller understanding of environmental stressors' biological impacts. This research contributes to the growing evidence of light pollution's profound influence on biological rhythms. Further exploration should focus on the mechanism of social amplification and consequences of disrupted circadian rhythms in group-living species. Additionally, future research should determine if sensitive individuals are the propagators of ALAN’s affects by grouping more and less sensitive individuals separately.

Together, these studies offer a comprehensive view of circadian rhythm disruption in genes, brains, and behavior, highlighting the resilience and vulnerabilities of biological clocks to environmental changes and pointing towards strategies for maintaining circadian homeostasis in an ever-changing world.