**Artificial night at light differentially affects birds in isolation or social conditions**

Cassandra K. Hui1\*, Yong Zhang2, Jenny Q. Ouyang1

1 Department of Biology, University of Nevada, Reno

1664 N Virginia St, Reno, NV 89557

2 Department …

\*Corresponding author: chui@nevada.unr.edu

**Abstract**

**Introduction**

Organisms rely on internal 24-hour rhythms, known as circadian rhythms, to coordinate various physiological and behavioral processes. These rhythms synchronize with external cues, such as light and temperature, to maintain biological rhythms in alignment with the day-night cycle. However, artificial light at night (ALAN) disrupts these rhythms, leading to profound effects on organismal health and behavior (*1, 2*). Despite its significance as an environmental pollutant, much of the existing research has concentrated on isolated animal models.

Social interactions play a pivotal role in shaping physiology, including circadian regulation (*3*). Previous research has demonstrated that social interactions can restore arrhythmic behavior in birds (*4*), and increase circadian gene expression in insects (*5*). Therefore, our study aims to investigate whether social conditions, akin to those in natural habitats, could mitigate the adverse effects of ALAN on birds.

In this study, we examined zebra finches (*Taeniopygia guttata*) exposed to ALAN in both isolated and social environments. We compared their activity levels, melatonin secretion, and circadian gene expression in the hypothalamus and liver to birds in control dark night conditions, housed in either isolated or social settings. We chose these metrics for a comprehensive analysis of behavioral, physiological, and molecular clock changes in response to ALAN, aiming to explore core and peripheral mechanistic clock changes and their interactions. We predict that socially interacting birds exposed to ALAN will exhibit fewer negative effects compared to their isolated counterparts.

**Methods**

*Experimental Design*

Zebra finches were caged indoors individually (47 x 31 x 36cm cages) or grouped (47 x 93 x 36 cm cages) and entrained 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 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 across cages. We also video recorded cages containing groups of birds every half hour for two minutes (6).

Birds were randomly assigned to one of four conditions: social ALAN (12L:12L dim), isolated ALAN (12L:12L dim), social control (12L:12D), and isolated control (12L:12D). As determined by One-Way ANOVA, groups did not differ in initial mass (p= 0.247). After the 3-week entertainment period, we sacrificed the birds at four-time points: ZT 1, ZT 7, ZT 13, and ZT19. ALAN was standardized to around 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 (*6*).

To acquire more melatonin data, we repeated the experiment with collecting 4 timepoints of blood throughout the day of each bird. After 9 days of ALAN exposure, we bleed birds (no more than 1% of body weight per 48 hours) at 3 different timepoints over 10 days. On the last day we sacrificed birds at their last timepoint and collected blood.

*Real-Time qPCR*

Real-time PCR quantification was based on SYBR-Green. Circadian gene expression in the hypothalamus was detected by dissection of the hypothalamus, homogenized, and analyzed in triplicate for technical repeats. Total RNA was isolated from collected tissues using Trizol (Life Technologies, Carlsbad, California) and quantified using Nanodrop 1000 (Thermo Scientific). Reverse transcription was done from 3 mg of total RNA through Versco cDNA synthesis kit. The primers were designed using Primer 3 based on Zebra Finch Cry1, Bmal1, Per2, and Per3 genes (Table S1). Amplicon abundance was calculated using the 2-∆∆CT method.

*Endocrinology*

Melatonin was measured from plasma using an enzyme-linked immunoassay kit (Aviva Systems Biology OKEH02566) on two 96-well plates according to manufacturer procedures. When available, 25 mL of plasma was diluted and used for each individual run in duplicates, samples with less than 50 mL of plasma were diluted more. 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). Optical density absorbance (OD) was contrasted from mean blank OD to obtain relative sample OD and then regressed against a standard curve to calculate final melatonin concentrations.

*Statistical Analyses*

Data and graphs were analyzed using R version 4.1.2 (R Development Core Team, 2019) and models were built to test for the effect of ALAN on activity, melatonin, and circadian gene rhythmicity. 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. with 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 cardiac gene expression at each timepoint. CircaCompare (version 0.1.1) was used for rhythmic analysis of gene expression. We used liner regression models to compare gene expression with melatonin and activity onset.

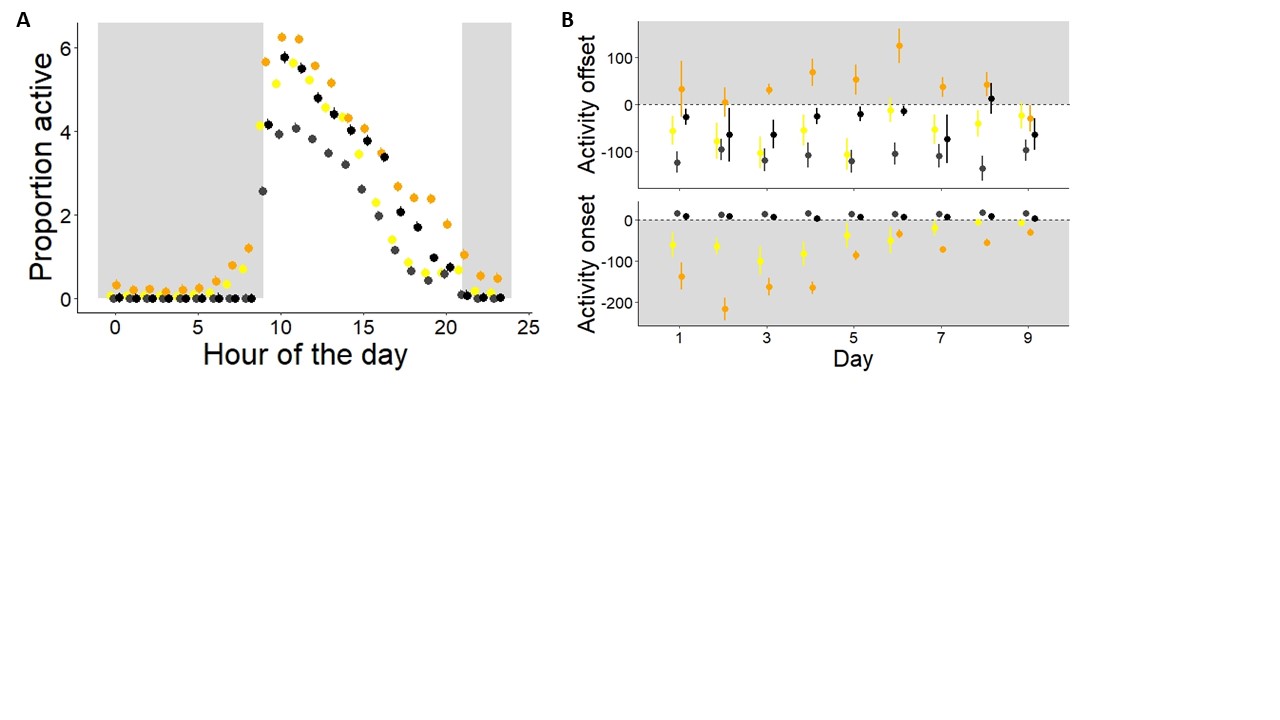
*Ethics Statement*

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.

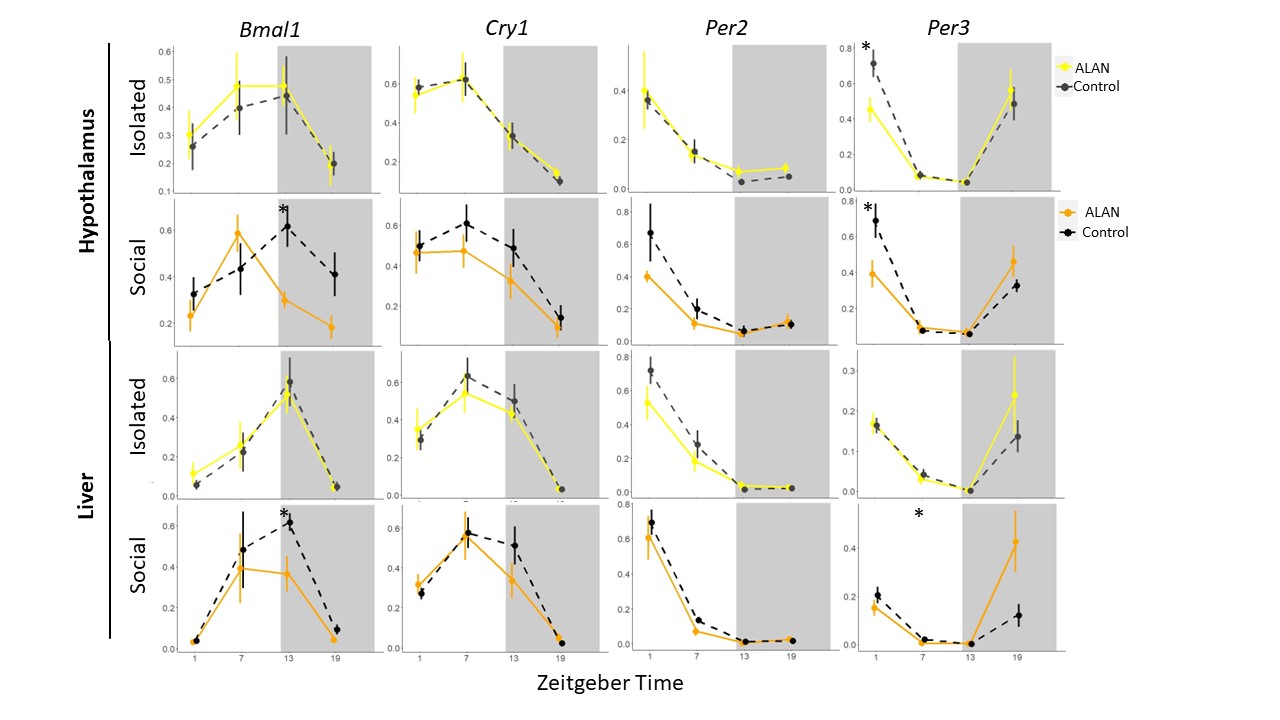
**Results**

*Activity*

Exposure to artificial light at night (ALAN) significantly increased nocturnal activity in zebra finches, unaffected by whether birds were housed individually or in groups. 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: p < 0.01, Social: p < 0.0) and lengthened their offset (Isolated: p < 0.01, Social: p < 0.01) compared to their controls.



**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. Data are reported as mean ± SEM. (B) Comparisons of activity onset and offset times between isolated and social conditions under ALAN exposure and control settings. Data are reported as mean ± SEM.



**Figure 2. Daily circadian gene expression in the hypothalamus and liver under ALAN.** Normalized expression of *Bmal1, Cry1, Per2,* 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 were significantly different from social controls in *Bmal1* expression at ZT 13 (p = 0.03) and *Per3* expression at ZT 7 (p = 0.05) in the liver. Significance stars: ‘\*’ p < 0.05, ‘\*\*’ p < 0.01, ‘\*\*\*’ p < 0.001.

*Gene Expression*

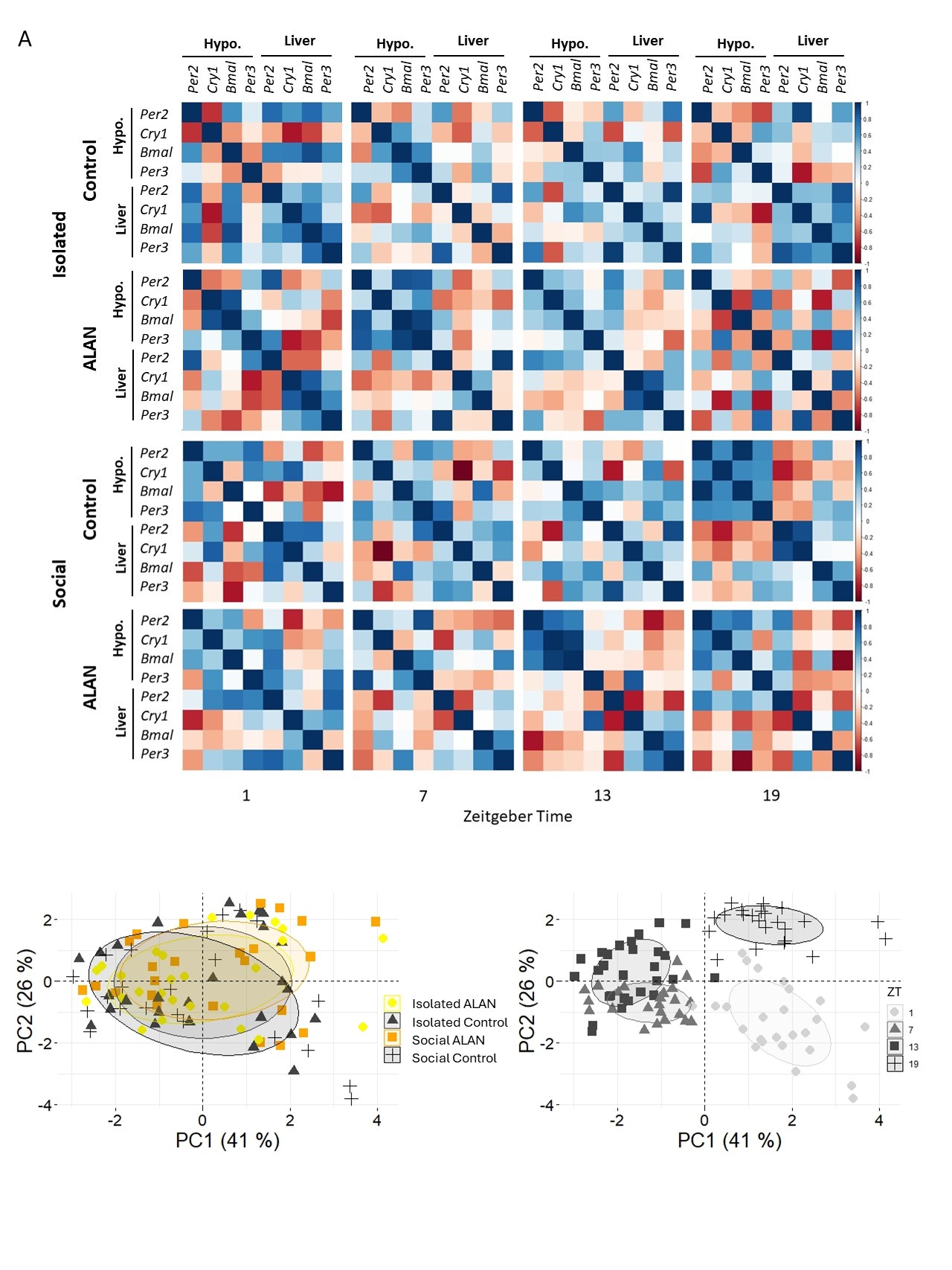
Consistent daily rhythms were observed in the expression of circadian genes *Cry1, Per2,* and *Per3* within the hypothalamus across all treatments (all p-values < 0.01: Figure 2). In contrast, *Bmal1* expression exhibited less pronounced rhythmicity, with variability observed across different conditions (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 1). A significant phase shift in *Bmal1* expression was identified 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 4), 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).

**Table 1.** Rhythmic analysis comparing difference in phase of expression of four circadian genes in the hypothalamus between birds exposed to ALAN and controls either in isolation or social conditions. Four timepoints were collected throughout the day at ZT 1, 7, 13, and 19.

|  |  |  |  |
| --- | --- | --- | --- |
| **Condition** | **Gene** | **Difference** | **p-value** |
|  | *Bmal1* | 0.70 | 0.77 |
| Isolated | *Cry1* | -0.14 | 0.89 |
|  | *Per2* | 0.61 | 0.63 |
|  | *Per3* | 1.20 | 0.08 |
|  | *Bmal1* | 5.00 | **0.01** |
| Social | *Cry1* | 1.44 | 0.36 |
|  | *Per2* | 0.96 | 0.55 |
|  | *Per3* | 1.70 | 0.05 |

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: Figure 5). Comparisons of phase and amplitude between control groups and those exposed to ALAN showed no significant differences for *Bmal1, Cry1,* and *Per2* expressions. Notably, 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 in controls and exposed to ALAN. 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.

A correlation analysis was conducted to examine the relationship between gene expression levels in the hypothalamus and liver among birds subjected to ALAN exposure and their corresponding controls, across both isolated and social conditions, at every timepoint (Figure 3A). A comprehensive Principal Component Analysis (PCA) of all gene expression data revealed that samples grouped according to timepoint rather than by exposure condition (Figure 3B).

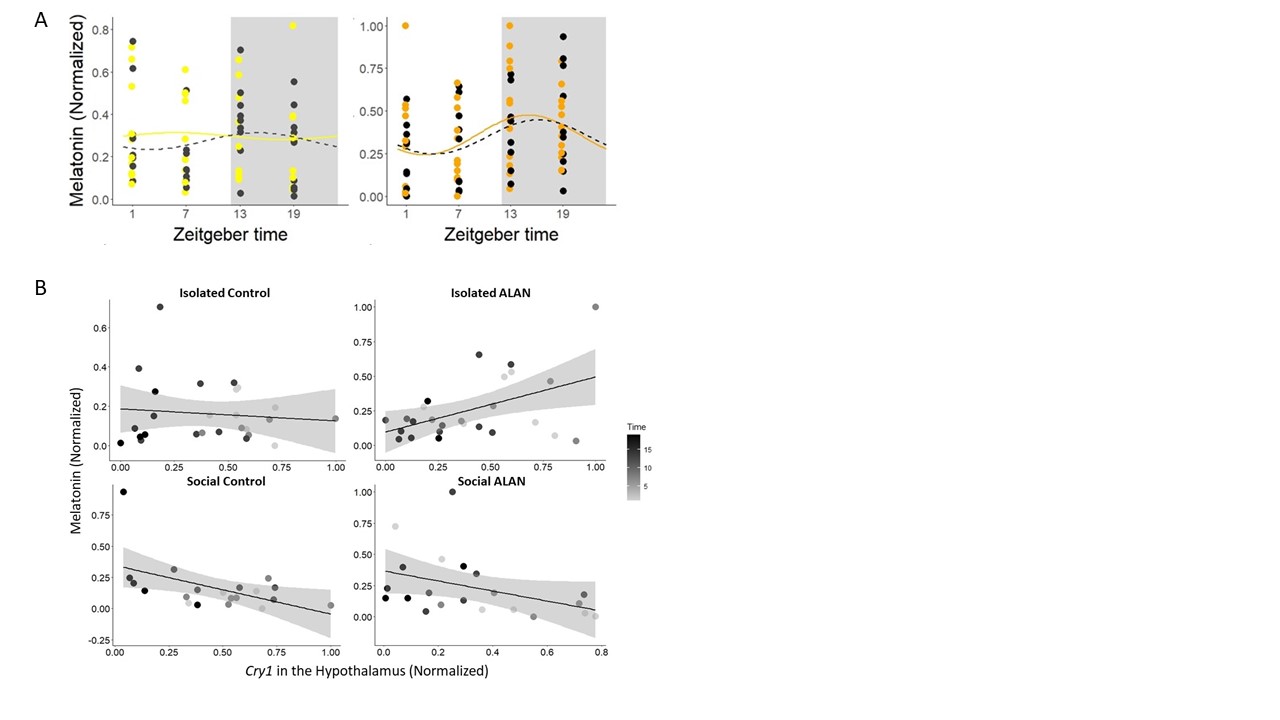


**Figure 3. Correlation matrix and PCA between circadian genes expressed in in the hypothalamus and liver under ALAN in isolation or social conditions.** (A) Matrixes are separated by control or exposre to ALAN and zeitgeber time (1, 7, 13 and 19). Dark blue shows a strong positive correlation and dark red shows a strong negative correlation between four circadian genes (*Bmal1, Cry1, Per2,* and *Per3*) in the hypothalamus and liver. (B) PCA plots of all gene expression in the hypothalamus and liver. Points represent individuals which cluster by timepoint and not treatment, elipsies cover 80% of samples.

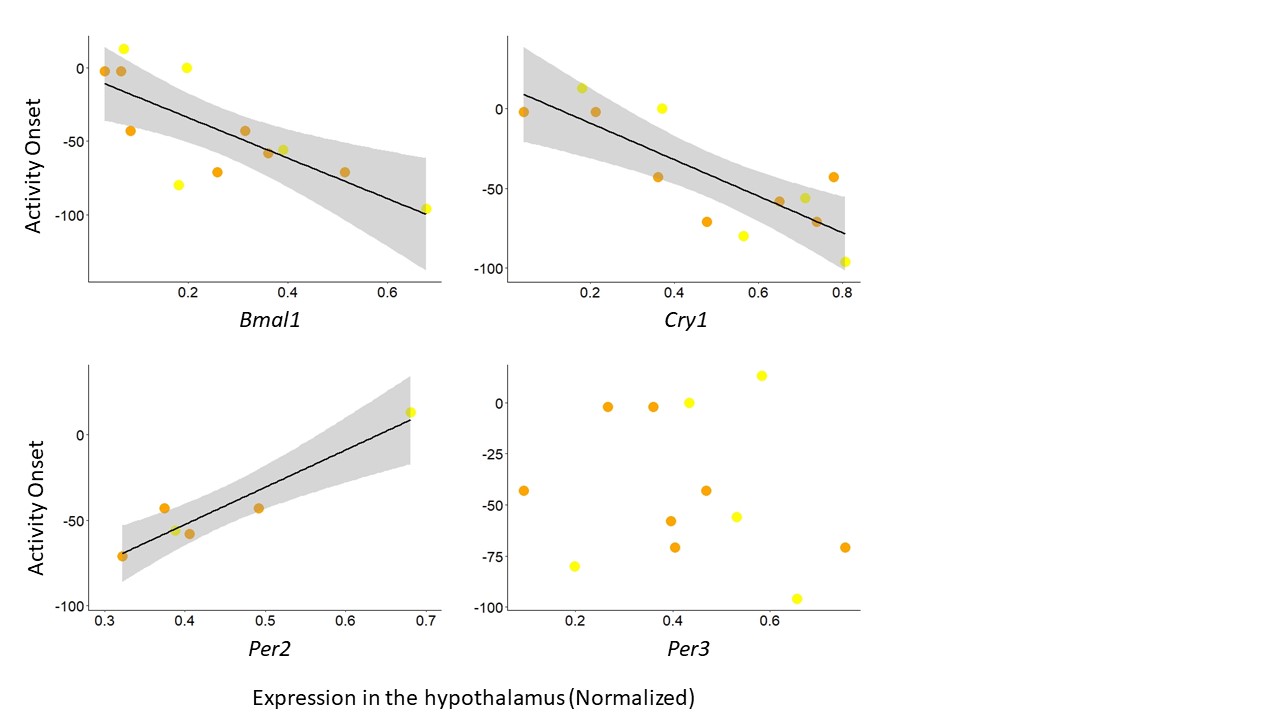
*Melatonin*

Melatonin concentrations oscillated throughout the day in all groups, peaking at night for isolated control and social birds, but during the day for isolated birds exposed to ALAN (Figure 4A). 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 also did not differ between birds exposed to ALAN regardless of social conditions.

*Gene expression and melatonin*

In the hypothalamus, *Cry1* expression exhibited a slight negative correlation with melatonin levels across all treatments, except for isolated birds under ALAN, where a moderate positive correlation was observed (Figure 4B). Linear regression analyses were conducted separately for isolated and socially house birds. For isolated birds, hypothalamic *Cry1* expression moderately correlated with melatonin levels (t = 1.84, p = 0.07) and similarly moderately associated with ALAN exposure (t = 1.78, p = 0.08). Conversely, within social housing conditions, *Cry1* expression strongly correlated with melatonin levels (t = -3.07, p < 0.01), yet showed no significant correlation with ALAN exposure (t = -0.25, p = 0.81).

**Figure 4. Daily melatonin levels and interactions with hypothalamic *Cry1* under ALAN across social conditions.** (A) Circadian rhythm of melatonin with fitted cosine curve overlaid. Shaded portions represent nighttime (ZT 12-ZT 24). The left panel shows birds in isolation and the right panel shows birds in social conditions, dark grey is isolated and black is social control, yellow is isolated under ALAN, and orange is social under ALAN. Samples were collected after 10 days of ALAN exposure at ZT 1, ZT 7, ZT 13, and ZT 19. Significant differences were not detected between birds exposed to ALAN and control birds in either isolation or social groups. Points represent individuals. (B) Scatter plots of hypothalamic *Cry1* and melatonin levels normalized across timepoints (grey scale). The top left is isolated controls, top right is isolated birds under ALAN, bottom left is social controls, and bottom right is social birds under ALAN. Lines are fitted as linear regression model and shaded portions represent 95% confidence interval. Points represent individuals.



**Figure 5. Hypothalamic circadian genes at ZT 1 predict activity onset.** Circadian genes *bmal1* (p < 0.01), *cry1* (p < 0.01), and *per2* (p < 0.01) expressed in the hypothalamus at ZT 1 predict activity onset of the last experimental day in birds exposed to ALAN. *per3* (p = 0.58) does not. Lines are fitted with statistically significant linear regression models and shaded portions represent 95% confidence interval. Points represent individuals.

*Gene expression and activity*

Hypothalamic circadian genes (*Bmal1, Cry1,* and *Per2*) expressed at ZT 1 strongly predict (Figure 5; all p < 0.01) activity onset under ALAN, aside from *Per3* (p = 0.58). We did not analyze control birds which maintained an onset close to 0.

**Discussion**

Artificial light at night (ALAN) disrupts circadian rhythms in both gene expression and behavioral patterns. ALAN exposure led to increased nocturnal activity and activity onset across isolated and social conditions, consistent with previous studies (*7*). Initially, there was a notable early onset of activity during the first few days of ALAN exposure, gradually tapering off over time while maintaining a consistent activity period, lengthening the activity offset.

ALAN exposure disrupted *Per3* expression in the hypothalamus uniformly, whereas *Bmal1* expression was disrupted in the hypothalamus and liver specifically in social birds. Zebra finches are social creatures stressed by isolation (*8*). The discrepancy between circadian gene disruption may suggest additional stressors, like ALAN, are less impactful in an already stressful environment of isolation. Conversely, birds in socially enriched environments may exhibit heightened sensitivity to stressors, potentially amplifying their responses.

Additionally, we did see a correlation between earlier activity onset due to ALAN exposure and hypothalamic gene expression. Other studies have shown behavioral shifts absent of circadian gene shifts (*6, 9*). Although we did not see ALAN strongly impact gene expression compared to controls, we can point to its relationship with activity onset which was significantly changed. Sample size or individual variation may have blurred individual differences in gene expression.

We did not see any significant differences in melatonin level across treatment groups. This was likely due to extreme individual variation. Previous research has shown ALAN disrupts melatonin in birds (*10*), although we were unable to replicate this we did see melatonin trending towards a daytime peak in isolated ALAN exposed birds that was absent in social conditions. We did see however, that ALAN exposure in isolation led to a positive association with hypothalamic C*ry1* expression, which held a slight negative association in all other treatments. ALAN unleashes widespread effects throughout the brain (*11*). In isolation, ALAN may disrupt an underlying function that alters melatonin and *Cry1* expression similarly, undetectable by our measures of these functions separately or masked by individual variation.

The findings of this study shed light on the complex interplay between social conditions, neuronal signaling pathways, and sleep-wake regulation in response to ALAN exposure. Our results suggest that ALAN disrupts normal circadian patterns in birds, as evidenced by increased nocturnal activity and alterations in hypothalamic gene expression across social conditions. Conversely, social enrichment led to disrupted peripheral circadian gene expression in the liver but may possibly mediate melatonin disruptions. These results show the importance of accounting for social context experimental lab settings as results may otherwise be less applicable to natural life.

**Acknowledgments**

**Supplemental**

**Supplementary Table 1:** Primers sequences designed based on Zebra Finch *Cry1*, *Bmal, Per2*, *Per3,* and 18S genes for qPCR.

|  |  |  |
| --- | --- | --- |
| **Primer** | | **Sequence (5’- 3’)** |
| *Cry1* | forward | GGTCTTCTTGCAACTGTGCC |
| reverse | AGCTGAGCTCCTCCTGTACT |
| *Bmal1* | forward | ATGGCTGTCCAGCACATGAA |
| reverse | CACAGCCCACAACGAAAAGG |
| *Per2* | forward | AGCAAGACCTGATGCCTGTC |
| reverse | ACATCGGACGTGAACAA AA |
| *Per3* | forward | TTGTGGCCAAGGTGATTCCC |
| reverse | TGTCTCTGAGGTTTCTGGCG |
| *18S* | forward | GCCGCTAGAGGTGAAATTCTTA |
| reverse | CTTTCGCTCTGGTCCGTCTT |

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