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**Birds of a feather flock together: Light pollution’s effects differ by social context.**

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**Abstract**

Artificial light at night (ALAN), a growing pervasive pollutant, disrupts physiological and behavioral rhythms across organisms. Social interactions could alter responses to stressors like ALAN however, social contexts have been largely overlooked, despite their significant role in shaping the daily lives and internal biological rhythms. 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 controls 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 mimic natural conditions better.

**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 (*1*). Organisms across taxa, including humans, synchronize biological rhythms with external cues, such as light and temperature, to maintain alignment with the day-night cycle. At the heart of these rhythms is the circadian clock, governed by a feedback loop of oscillating core genes. Clock (*Clk*) and Brain and muscle Arnt-like protein-1 (*Bmal1*) genes promote Period (*Per*) and Cryptochrome (*Cry*) expression which in turn repress their own activity (*2*). This system is entrained to environmental cues, primarily by the degradation of the PER/CRY protein complex in light (*2*). The main clock, in the suprachiasmatic nucleus (SCN) nestled in the hypothalamus, coordinates peripheral clocks in other tissues, such as the liver, which can also entrain downstream physiology and behaviors, like hormone secretion and activity periods (*3*).

Melatonin, produced by the pineal gland during the night, serves as a critical signal for sleep readiness and regulates various biological functions as it aligns with the night-day cycle (*4*). Its production, tightly controlled by the circadian clock, forms a vital link between the external environment's light-dark cycle and the organism's internal biological processes.

Despite the established disruptive effects of ALAN on a wide variety of circadian regulation from neuronal activity to behavior (*5-7*), much of the existing research has concentrated on isolated animal models or housing conditions have largely been ignored (*7, 8*). 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 (*9-12*). Therefore, our study aims to investigate whether social conditions, akin to those in natural habitats, alter the effects of ALAN through mitigation or exacerbation.

We exposed zebra finches (*Taeniopygia guttata*), a social diurnal model organism, 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 would be less in ALAN-exposed birds housed socially than isolated conditions. Alternatively, ALAN could be a strong enough *zeitgeber* or stressor that the social context has no effect. In this case, ALAN exposure would elicit similar responses regardless of social condition.

**Methods**

*Experimental Design*

Ninety-nine zebra finches were caged indoors individually (n=53; 47 x 31 x 36cm cages) or grouped (n=46, 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 (*13*).

Birds were randomly assigned 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 (*8*). As determined by One-Way ANOVA, groups did not differ in initial mass (p= 0.25). After the 3-week entertainment period birds were exposed to ALAN 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 blood samples at four different times (ZT 1, ZT 7, ZT 13, and ZT 19) per bird. 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. 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 *Cry1*, *Bmal1*, *Per2*, and *Per3* genes (Table S1). Amplicon abundance was calculated using the 2-∆∆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. 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 calculated interplate coefficient of variation (CV) for our repeated standard was 9.5% for the first round and 3.2% for the second. The intraplate CV was 2.8% for the first and 7.8% for the second round. To increase accuracy, we normalized melatonin levels within each experimental round.

*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 liner regression models to compare gene expression with melatonin and activity onset. All models met assumptions and significance was taken at α=0.05.

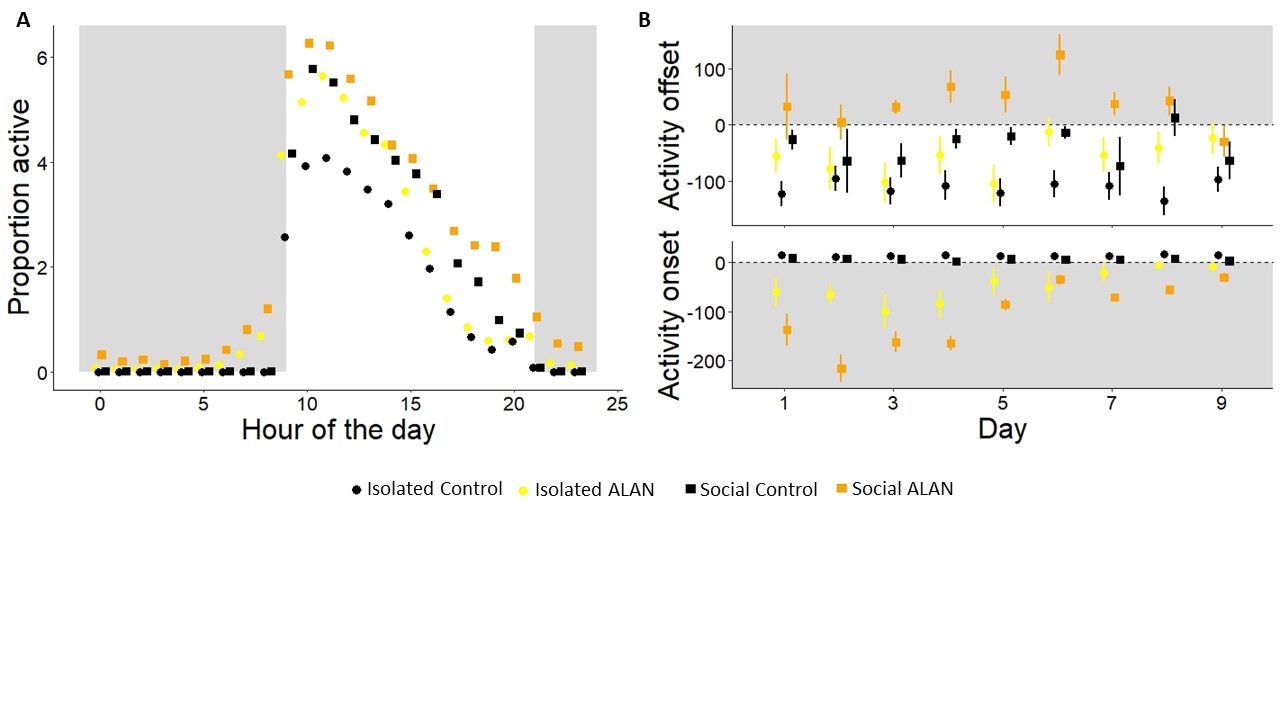
*Ethics Statement*

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.

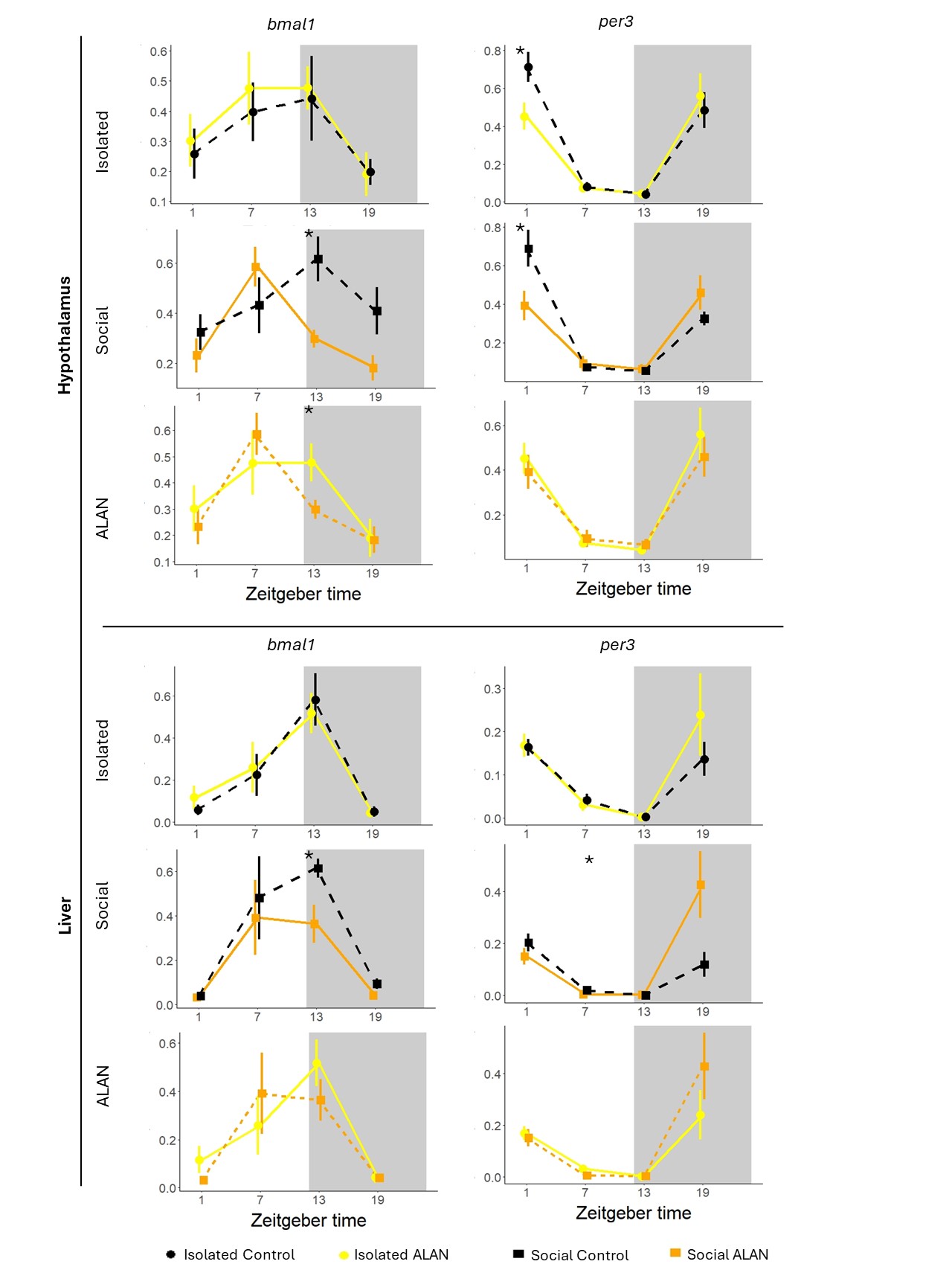
**Results**

*Activity*

Exposure to artificial light at night (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: p < 0.01, Social: p < 0.01) and lengthened their offset (Isolated: p < 0.01, Social: p < 0.01) compared to their controls. However, social birds had a significantly earlier activity onset than isolated birds when exposed to ALAN (p < 0.01).



**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* 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. Asterisks: ‘\*’ p < 0.05, ‘\*\*’ p < 0.01, ‘\*\*\*’ p < 0.001.

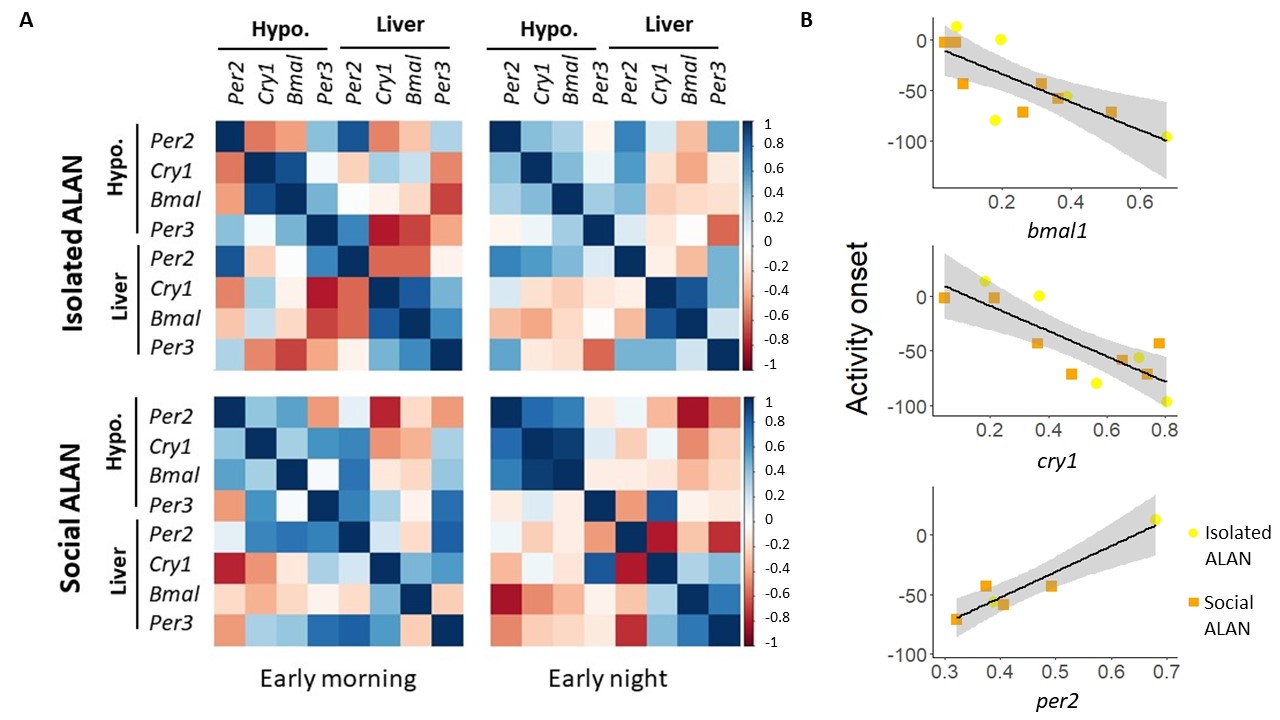
*Gene Expression*

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). 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. 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 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).

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. 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). 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.



**Figure 3. Correlation matrix of circadian genes expressed in the hypothalamus and liver under ALAN and predition of acticty onset.** (A) Matrixes are separated by isolated or socially housed birds and time (early mrodning 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.

*Melatonin*

Next, we measured melatonin levels to determine if the altered circadian genes were disrupting 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.

**Discussion**

Our research examined the impact of ALAN on zebra finches, exploring how these effects may be modulated by social interaction. Our results corroborate existing literature, by showing ALAN significantly disrupts circadian rhythms (*14*), and extends it by demonstrating that social contexts amplify the disruptions caused by ALAN, particularly in behavioral patterns and rhythmic gene expression.

ALAN disrupts circadian rhythms in both behavioral patterns and gene expression which is consistent with previous studies (*1, 15*). 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.

The core clock in the SCN synchronizes peripheral clocks throughout an organism (*16*), and misalignment causes disease (*17*). 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 (*18, 19*). We found that ALAN reorganized the relationship of circadian genes differently in birds that were isolated compared to social. 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.

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. This finding diverges from some prior studies reporting ALAN-induced melatonin disruption in birds housed individually or socially (*7, 20, 21*). This was likely due to extreme individual variation in our samples demonstrating the complex interplay of physiological responses to environmental changes.

In social settings, organisms often synchronize their activities and physiological processes for various benefits, including enhanced cohesion and survival (*22-24*). The synchronization of circadian rhythms, facilitated by social cues, is a critical aspect of this 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 (*10*). 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 pollutions. This has significant implications for understanding the ecological impacts of ALAN, as social dynamics and group living are common across many species especially humans. 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.

**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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