Look for visual patterns, don't worry about the “control”. Separate by timepoints and treatments. Color by other factors.

Liver gene expression is very different in SLD from LD:

> summary(model)

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']

Formula: norm\_mel ~ Lpc1\_scores \* treatment \* time + (1 | Cage)

Data: com\_dat2

REML criterion at convergence: 59.3

Scaled residuals:

Min 1Q Median 3Q Max

-1.4891 -0.5745 -0.2585 0.3609 3.6202

Random effects:

Groups Name Variance Std.Dev.

Cage (Intercept) 0.00000 0.0000

Residual 0.04252 0.2062

Number of obs: 94, groups: Cage, 22

Fixed effects:

Estimate Std. Error df t value Pr(>|t|)

(Intercept) 0.1798529 0.0739674 78.0000000 2.432 0.0173 \*

Lpc1\_scores -0.0323983 0.0475006 78.0000000 -0.682 0.4972

treatmentA 0.1674862 0.1066385 78.0000000 1.571 0.1203

treatmentSA 0.0034679 0.1087399 78.0000000 0.032 0.9746

treatmentSLD -0.2477566 0.1199703 78.0000000 -2.065 0.0422 \*

time -0.0028764 0.0065038 78.0000000 -0.442 0.6595

Lpc1\_scores:treatmentA 0.0560409 0.0756512 78.0000000 0.741 0.4611

Lpc1\_scores:treatmentSA 0.0130102 0.0852546 78.0000000 0.153 0.8791

Lpc1\_scores:treatmentSLD -0.1132922 0.0879681 78.0000000 -1.288 0.2016

Lpc1\_scores:time 0.0005113 0.0039323 78.0000000 0.130 0.8969

treatmentA:time -0.0060118 0.0091563 78.0000000 -0.657 0.5134

treatmentSA:time 0.0082716 0.0093072 78.0000000 0.889 0.3769

treatmentSLD:time 0.0249275 0.0102207 78.0000000 2.439 0.0170 \*

Lpc1\_scores:treatmentA:time -0.0022857 0.0065517 78.0000000 -0.349 0.7281

Lpc1\_scores:treatmentSA:time 0.0023910 0.0067831 78.0000000 0.352 0.7254

Lpc1\_scores:treatmentSLD:time 0.0175939 0.0076587 78.0000000 2.297 0.0243 \*

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Signif. codes: 0 ‘\*\*\*’ 0.001 ‘\*\*’ 0.01 ‘\*’ 0.05 ‘.’ 0.1 ‘ ’ 1

In our study, we observed that while there were no significant differences in CRY gene expression or melatonin levels between birds exposed to natural light-dark cycles (LD) and those subjected to artificial light at night (A), the relationship between CRY gene expression and melatonin secretion diverged significantly across these groups. This intriguing finding suggests a complex modulation of the circadian regulation mechanism, where the direct impact of artificial light does not manifest in altered levels of CRY or melatonin independently but rather in the nuanced interaction between these circadian components. The differential relationship under artificial lighting conditions could reflect an altered sensitivity of the circadian feedback loop, potentially affecting the timing and synchronization of biological processes regulated by the circadian clock. Despite similar expression levels of CRY, its functional role in signaling and regulating melatonin secretion appears to be modified in the presence of artificial light, hinting at a subtler form of circadian disruption that standard measurements of gene expression and hormone levels may not fully capture. This observation underscores the importance of examining the functional dynamics of circadian regulation, beyond mere quantification of its molecular constituents, to understand the biological ramifications of artificial lighting on circadian health and organismal well-being.

There is a difference between brain PC1 for social group with treatment and time

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| Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']  Formula: norm\_mel ~ Bpc1\_scores \* treatment \* time + (1 | Cage)  Data: soc  REML criterion at convergence: 32.5  Scaled residuals:  Min 1Q Median 3Q Max  -1.1788 -0.6108 -0.2537 0.3134 3.1018  Random effects:  Groups Name Variance Std.Dev.  Cage (Intercept) 0.004811 0.06936  Residual 0.045676 0.21372  Number of obs: 42, groups: Cage, 3  Fixed effects:  Estimate Std. Error df t value Pr(>|t|)  (Intercept) 0.100020 0.104879 3.836668 0.954 0.396  Bpc1\_scores 0.068396 0.079754 28.665240 0.858 0.398  treatmentSLD -0.099468 0.142210 16.276357 -0.699 0.494  time 0.012456 0.008966 28.923127 1.389 0.175  Bpc1\_scores:treatmentSLD -0.139727 0.103963 30.894598 -1.344 0.189  Bpc1\_scores:time -0.008975 0.008838 28.197024 -1.016 0.318  treatmentSLD:time 0.004716 0.011876 28.764837 0.397 0.694  Bpc1\_scores:treatmentSLD:time 0.021999 0.011099 28.995211 1.982 0.057 .  ---  Signif. codes: 0 ‘\*\*\*’ 0.001 ‘\*\*’ 0.01 ‘\*’ 0.05 ‘.’ 0.1 ‘ ’ 1  Correlation of Fixed Effects:  (Intr) Bpc1\_s trtSLD time Bp1\_:SLD Bpc1\_: trSLD:  Bpc1\_scores -0.371  treatmntSLD -0.672 0.267  time -0.755 0.534 0.549  Bpc1\_sc:SLD 0.265 -0.765 -0.074 -0.407  Bpc1\_scrs:t 0.434 -0.872 -0.316 -0.671 0.668  trtmntSLD:t 0.575 -0.404 -0.795 -0.756 0.193 0.507  Bpc1\_s:SLD: -0.335 0.693 0.120 0.533 -0.828 -0.796 -0.292 |
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ALAN birds have a sigficantly longer activity period  
  
> post\_hoc\_result

Tukey multiple comparisons of means

95% family-wise confidence level

Fit: aov(formula = act\_length ~ treatment, data = dat\_long)

$treatment

diff lwr upr p adj

LD-A -116.99056 -154.91746 -79.063663 0.0000000

SA-A 168.30695 130.92130 205.692604 0.0000000

SLD-A -31.56502 -68.67865 5.548606 0.1269278

SA-LD 285.29751 248.72223 321.872797 0.0000000

SLD-LD 85.42554 49.12834 121.722727 0.0000000

SLD-SA -199.87198 -235.60324 -164.140707 0.0000000

> summary(model)

Call:

lm(formula = on9 ~ treatment \* Bpc1\_scores, data = subset(com\_dat2,

time == 7))

Residuals:

Min 1Q Median 3Q Max

-19.590 -11.292 0.066 8.586 35.408

Coefficients:

Estimate Std. Error t value Pr(>|t|)

(Intercept) 40.003 9.560 4.184 0.00107 \*\*

treatmentA -23.178 12.229 -1.895 0.08051 .

treatmentSA -94.829 12.106 -7.833 2.81e-06 \*\*\*

treatmentSLD -29.311 12.206 -2.401 0.03200 \*

Bpc1\_scores 11.038 4.360 2.532 0.02503 \*

treatmentA:Bpc1\_scores -1.702 9.146 -0.186 0.85526

treatmentSA:Bpc1\_scores -6.954 5.738 -1.212 0.24707

treatmentSLD:Bpc1\_scores -12.651 7.579 -1.669 0.11895

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Signif. codes: 0 ‘\*\*\*’ 0.001 ‘\*\*’ 0.01 ‘\*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 15.88 on 13 degrees of freedom

(2 observations deleted due to missingness)

Multiple R-squared: 0.8746, Adjusted R-squared: 0.8071

F-statistic: 12.95 on 7 and 13 DF, p-value: 6.027e-05

In our study, we investigated the influences of environmental treatments and brain gene expression on the timing of activity onset in birds, focusing on a critical time point at ZT 7. Our analysis revealed significant correlations between both treatment types and brain gene expression levels with activity onset times, underscoring the complex interplay between external environmental cues and internal genetic mechanisms in regulating circadian behaviors.

Specifically, we found that different treatments exerted varying degrees of influence on activity onset. Notably, birds subjected to treatment SA exhibited a substantial advancement in their activity onset, beginning their daily activities approximately 95 minutes earlier than those in the reference group. This pronounced effect highlights the significant impact that specific environmental conditions can have on circadian rhythms. Conversely, treatment A also influenced activity onset, albeit to a lesser extent, indicating a gradient of responsiveness across the treatment spectrum.

Moreover, our analysis shed light on the role of brain gene expression in modulating activity onset. Higher levels of certain brain gene expressions were associated with a delayed activity onset, suggesting an intrinsic genetic component that tempers the birds' response to external cues. This finding points to the genetic underpinnings of circadian regulation, where the activity of specific genes can influence the timing of behavioral responses to the day-night cycle.

Importantly, while both treatment and brain gene expression independently correlated with activity onset, our study did not find a significant interaction between these factors at the examined time point. This suggests that the effects of environmental treatments on activity onset operate independently of the variations in brain gene expression levels we measured. Such a distinction underscores the multifactorial nature of circadian regulation, involving both adaptable responses to the environment and inherent genetic predispositions.

In summary, our findings highlight the dual influence of external treatments and internal genetic factors on circadian behavior, with both elements independently contributing to the determination of activity onset times in birds. These insights into the regulatory mechanisms of circadian rhythms underscore the intricate balance between environmental adaptability and genetic programming in the orchestration of daily biological rhythms.

Our analysis revealed nuanced relationships between hypothalamic cry1 gene expression and melatonin levels across different experimental conditions. Specifically, we observed a distinct interaction effect between social context, ALAN exposure, and cry1 expression in regulating melatonin levels.

In isolated conditions, the correlation between cry1 expression and melatonin levels tended towards a moderate positive direction under ALAN exposure, although this relationship did not reach statistical significance (t = 1.84, p = 0.07). This suggests a potential for increased cry1 expression to be associated with elevated melatonin levels in the absence of social interactions, particularly under conditions of artificial light at night (ALAN). Additionally, ALAN exposure itself showed a trend towards influencing melatonin levels (t = 1.78, p = 0.08), indicating a possible direct effect of light exposure on circadian regulation in isolated individuals.

Contrastingly, within social groups, cry1 expression demonstrated a strong and statistically significant negative correlation with melatonin levels (t = -3.07, p < 0.01). This robust relationship underscores the influential role of cry1 in the hypothalamus as a predictor of melatonin expression in a social context, where increased gene expression is associated with decreased melatonin levels. Notably, the direct effect of ALAN exposure on melatonin levels was not significant in social conditions (t = -0.25, p = 0.81), suggesting that the social environment may buffer or override the direct effects of light exposure on circadian hormone levels.

These findings underscore the complex interplay between gene expression, environmental factors, and social context in the regulation of circadian rhythms. While cry1 expression in the hypothalamus appears to play a pivotal role in predicting melatonin levels, the direction and strength of this relationship are markedly influenced by the presence of social interactions and environmental conditions such as ALAN exposure. Our results highlight the importance of considering both social and environmental contexts when investigating the mechanisms underlying circadian regulation.

MELATONIN DISRUPTED IN ISO ALAN?

No correlations with PC1 and mel (except all at ZT19) (At ZT 19 mel correlated with liver genes (except L\_BMAL) but not brain genes) (At ZT13 mel positively correlates with PER2 and PER3 in the liver, and at ZT1 mel positively correlates with CRY and BMAL in the liver) At ZT1 mel negatively correlates with brain genes.

Individual genes:

Melatonin almost correlates with **BMAL** (brain) in SA (p=0.06207) but not any other group (p~0.9).

Melatonin and **CRY**: SLD: p=0.02, SA: p=0.07, **LD: p=0.6**, A: p= 0.01 (only one that goes up)

Nothing with PER2 or PER3

**Figure 3. Melatonin in relation to hypothalamic circadian gene expression in birds exposed to ALAN in either isolation or 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 ALAN, and orange is social 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. (B) Correlation between melatonin and *cry1* mRNA expression in the hypothalamus. Isolated birds exposed to ALAN have a significant positive correlation (p=0.01) not seen in other treatment groups. (C) Correlation between melatonin and the first principal component of a PCA of four circadian genes expressed in the hypothalamus during the night. Birds exposed to ALAN have a negative correlation (A: p=0.06).

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| post\_hoc\_result  Tukey multiple comparisons of means  95% family-wise confidence level  Fit: aov(formula = act\_length ~ treatment, data = dat\_long)  $treatment  diff lwr upr p adj  LD-A -116.99056 -154.91746 -79.063663 0.0000000  SA-A 168.30695 130.92130 205.692604 0.0000000  SLD-A -31.56502 -68.67865 5.548606 0.1269278  SA-LD 285.29751 248.72223 321.872797 0.0000000  SLD-LD 85.42554 49.12834 121.722727 0.0000000  SLD-SA -199.87198 -235.60324 -164.140707 0.0000000 |
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| Tukey multiple comparisons of means  95% family-wise confidence level  Fit: aov(formula = on ~ treatment, data = dat\_long)  $treatment  diff lwr upr p adj  LD-A 65.22713 43.33386 87.12041 0.0000000  SA-A -66.65158 -88.22253 -45.08062 0.0000000  SLD-A 56.93713 35.52823 78.34604 0.0000000  SA-LD -131.87871 -153.32084 -110.43658 0.0000000  SLD-LD -8.29000 -29.56910 12.98910 0.7475852  SLD-SA 123.58871 102.64138 144.53604 0.0000000 |
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| > post\_hoc\_result  Tukey multiple comparisons of means  95% family-wise confidence level  Fit: aov(formula = off ~ treatment, data = dat\_long)  $treatment  diff lwr upr p adj  LD-A -53.92119 -80.169171 -27.67321 0.0000009  SA-A 98.91972 73.200880 124.63856 0.0000000  SLD-A 20.94750 -4.771342 46.66634 0.1549816  SA-LD 152.84091 127.692350 177.98947 0.0000000  SLD-LD 74.86869 49.720128 100.01725 0.0000000  SLD-SA -77.97222 -102.567998 -53.37645 0.0000000 |
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