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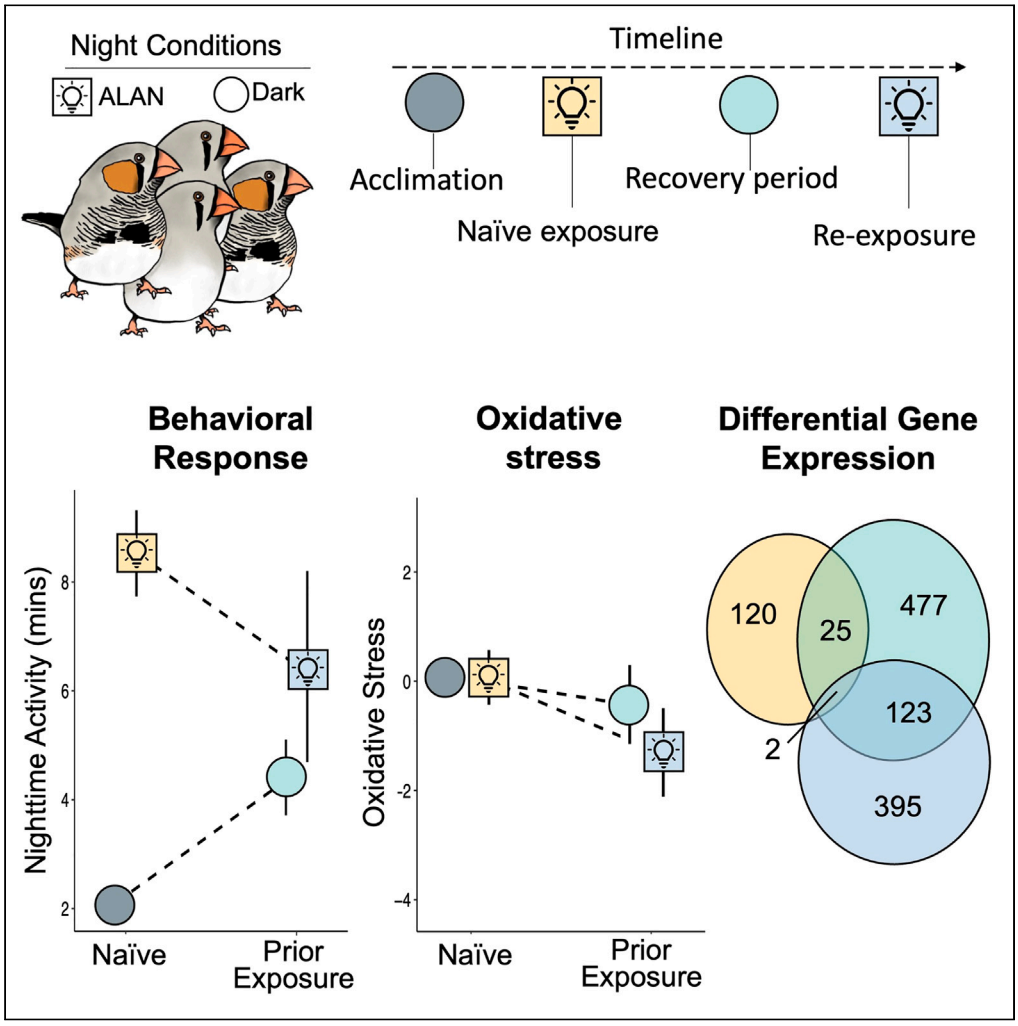

Valentina J. Alaasam, Cassandra Hui, Johnathan Lomas, Stephen M. Ferguson, Yong Zhang, Won Cheol Yim, Jenny Q. Ouyang

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Highlights

Zebra finches show behavioral habituation to repeated light pollution exposure

Slight decrease in oxidative stress detected after chronic (8 weeks) ALAN exposure

No detected differences among treatment groups in telomere attrition

Blood transcriptome reveals unique responses to naive, past, and repeated exposure

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What happens when the lights are left on? Transcriptomic and phenotypic habituation to light pollution

Valentina J. Alaasam,[1,5,*] Cassandra Hui,[1] Johnathan Lomas,[2] Stephen M. Ferguson,[3] Yong Zhang,[4] Won Cheol Yim,[2] and Jenny Q. Ouyang[1]

SUMMARY

Artificial light at night (ALAN) is a ubiquitous pollutant worldwide. Exposure can induce immediate behavioral and physiological changes in animals, sometimes leading to severe health consequences. Nevertheless, many organisms persist in light-polluted environments and may have mechanisms of habituating, reducing responses to repeated exposure over time, but this has yet to be tested experimentally. Here, we tested whether zebra finches (*Taeniopygia guttata*) can habituate to dim (0.3 lux) ALAN, measuring behavior, physiology (oxidative stress and telomere attrition), and gene expression in a repeated measures design, over 6 months. We present evidence of tolerance to chronic exposure, persistent behavioral responses lasting 8 weeks post-exposure, and attenuation of responses to re-exposure. Oxidative stress decreased under chronic ALAN. Changes in the blood transcriptome revealed unique responses to past exposure and re-exposure. Results demonstrate organismal resilience to chronic stressors and shed light on the capacity of birds to persist in an increasingly light-polluted world.

INTRODUCTION

Animals living in the Anthropocene are inundated with rapid, human-induced environmental changes, including habitat loss, invasive species, novel resources, and pollutants. Though some populations decline in altered environments, many persist and even thrive.[1,2] Those that persist often exhibit traits that are different from conspecifics in surrounding undisturbed habitats[3–5] suggesting rapid population-level phenotypic change. Understanding the mechanisms that enable these changes and facilitate species persistence in altered environments is thus a priority in one health initiatives and conservation.[6]

Habituation is one such mechanism, whereby individuals may adjust to novel environmental conditions via phenotypic plasticity or behavioral flexibility. Habituation can be defined as a reduction in behavioral and/or physiological responsiveness to repeated exposure.[7] Habituation is likely important for facilitating persistence through rapid environmental change because it allows individuals to respond immediately, reducing costs of a novel environment within one generation.[8] Nonetheless, habituation to anthropogenic change is not often documented because it is difficult to obtain repeated measures from the same individuals before and after discrete environmental change (but see[9,10]).

Artificial light at night (ALAN) dramatically alters the nighttime environment and has become one of the most ubiquitous human-induced environmental changes of our time, covering over 23% of the earth's land surface.[11] Across taxa, individuals exposed to ALAN show differences in behavior (e.g., timing and duration of sleep/wake cycles) and physiology (e.g., reduced melatonin, increased glucocorticoids),[12,13] which in some cases can lead to severe health consequences.[14,15] For example, complete circadian misalignment associated with nightshift work has been linked to depression, cardiovascular disease, metabolic disorders, and cancer.[16–20] However, contrary to bright levels of ALAN (representative of nightshift work), we have previously shown in zebra finches that dim levels of ALAN (<2 lux, representative of light pollution) induce a masking response,[21] where individuals change behavior immediately without participation of upstream circadian clock regulation. Because individuals can respond to dim ALAN via masking, we predict they may also be able to habituate to ALAN, reducing responses to subsequent exposure and minimizing long-term health consequences. However, most studies examining ALAN effects use bright levels of ALAN that disrupt circadian regulation and are limited to single, short-term exposures.[14,22–27] The capacity for animals to habituate to ALAN has yet to be tested. Furthermore, it is unknown whether physiological costs may accumulate because of repeated exposure long-term. Even if behavioral responses to dim ALAN are flexible,[27,28] physiological stress resulting either directly from ALAN exposure or as a byproduct of

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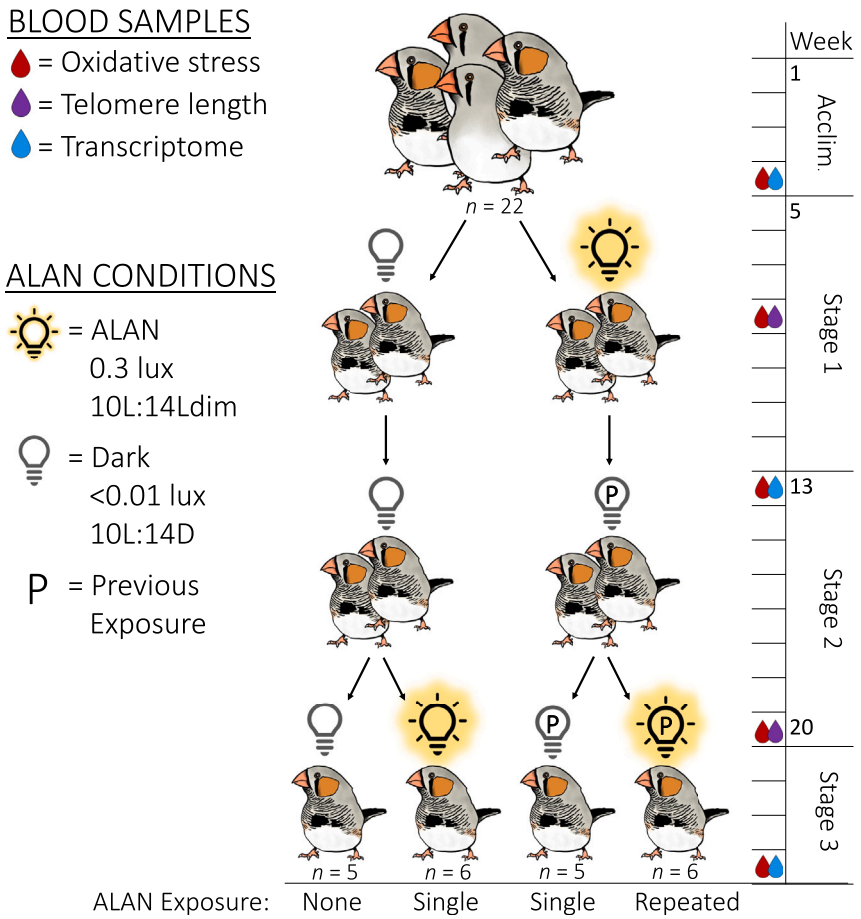

Figure 1. Experimental design and schedule

Zebra finches were divided into four groups. Controls were kept under dark night conditions for the entirety of the experiment (no ALAN exposure). Two groups experienced a single ALAN exposure, either in stage 1 or stage 3. Because we found no effect of captivity or time of year on behavioral responses to ALAN, these groups both represent single, naive exposures. The experimental group was exposed to ALAN in both stages 1 and 3 ('repeated' exposure), to test for habituation. Blood samples were collected at 5 timepoints. All plasma was used for oxidative stress analyses, and red blood cells were used for either telomere length or transcriptome analyses.

sleep debt might accumulate.[29] Indeed, moderate sleep debt is known to reduce oxidative defenses,[30,31] leading to hypertension and increased risk of cardiovascular diseases,[32,33] and accelerating telomere attrition.[34,35] Integrative research is needed because if individuals habituate behaviorally but incur physiological costs, then we may underestimate effects of ALAN based on behavioral observation alone.

We conducted a 6-month long experiment using zebra finches (*Taeniopygia guttata*) to test the capacity for birds to habituate to dim ALAN, and whether there is concordance between behavioral and physiological (oxidative stress and telomere attrition) responses (Figure 1). Oxidative stress is a common biomarker of long-term physiological stress[36] that is known to result from sleep debt, but is dynamic and reversible.[30,37] In contrast, telomere attrition reflects relatively irreversible physiological damage (in the absence of telomerase activity (32)) that is predictive of longevity,[38] and the rate of telomere attrition can increase in stressful environments.[39] We then used next-generation RNA sequencing to identify and quantify differentially expressed genes that may underly habituation (or lack thereof) to long-term and repeated ALAN exposure. We focused on the blood transcriptome to detect biomarkers of downstream responses related to physiological stress, oxidative damage, immune function, and pathology,[40,41] and to obtain repeated measures within individuals over time.

We predicted habituation would be characterized by an initial tolerance to chronic ALAN exposure followed by a reduction in response upon repeated exposure. First, we exposed individuals to chronic dim ALAN (8 weeks) and predicted that behavioral responses would either decrease or remain constant without concomitant increases in oxidative stress. Alternatively, a lack of tolerance could result in sensitization (i.e., increasing behavioral response) accompanied by increases in oxidative stress, ultimately leading to accelerated telomere attrition. Second, we tested whether individuals could accrue resistance to ALAN, reducing responses to a secondary re-exposure after a period of recovery. We predicted that if individuals habituate, the experience of previous exposure to ALAN would result in an attenuated behavioral response to re-exposure, and no increase in oxidative stress. Additionally, if birds habituate then we predicted to find more within-individual

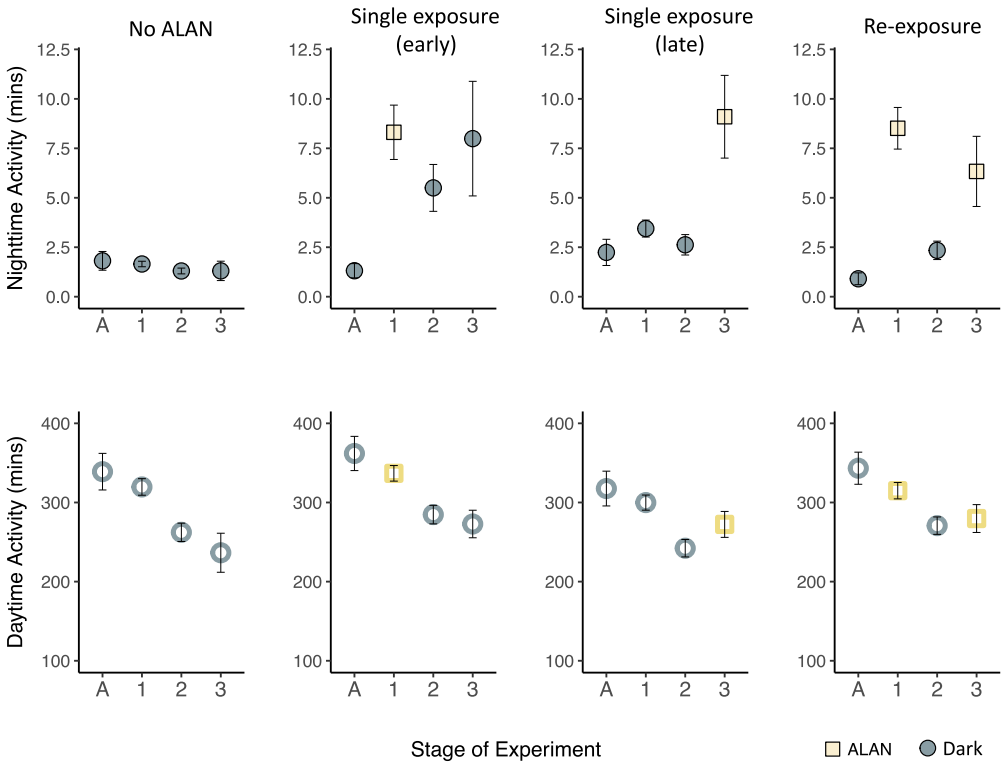

Figure 2. Effect of ALAN on activity

Nighttime (top, solid circle) and daytime (bottom, open circle) activity, measured as total minutes active in the night and/or day, across stages of the experiment. Stage "A" represents acclimation period. Gray circles indicate stages when groups were kept under normal 10L:14D conditions. Yellow squares indicate stages when different groups were exposed to dim (0.3 lux) ALAN (10L:14dim). Error bars represent standard error.

changes in gene expression after naive (first-time) exposures, compared to after re-exposures. We interpreted any repeatable changes in gene expression found in both naive and secondary exposures as repeatable responses to ALAN, and any unique changes as specific responses to re-exposure.

RESULTS

Behavior

The duration of our experiment was 23 weeks, divided into the following sections: initial acclimation, experimental stage 1 (ALAN exposure), experimental stage 2 (recovery period) and experimental stage 3 (ALAN exposure; Figure 1). First, we compared naive responses in experimental stages 1 (week 7) and 3 (week 23) to ensure there were no effects of time (or duration captivity) on ALAN responses that may confound later comparisons of first and secondary exposures. There was no effect of sex ($p = 0.63$, $n = 11$) on activity and so sex was not included in the final model. There was a significant effect of ALAN treatment (Table S1), where ALAN exposure increased nighttime activity by 407%. Under dark-night conditions birds were active 2.1 min per night (on average), compared to 8.5 min under ALAN. There was no difference in nighttime activity between stage 1 and stage 3 in either treatment condition (dark-night ($n = 11$): contrast $\pm$SE = 0.14 $\pm$ 0.17, adjusted-$p = 0.41$; ALAN-exposed ($n = 11$): contrast $\pm$SE = -0.24 ± 0.43, adjusted-$p = 0.58$). There was a decline in daytime activity over the duration of the experiment for all groups (slope $\pm$SE = $-0.002 \pm$ 3.26e-05, $p < 0001$, Figure 2; Figure S3).

In stage 1, we asked whether naive responses to ALAN depended on duration of exposure. ALAN increased nighttime activity, and this response did depend upon duration of ALAN exposure (Table 1 *top*; contrast $\pm$SE = -0.00427 ± 0.002, $p = 0.007$). There was no change in nighttime activity over time for birds under dark-night conditions (slope $\pm$SE = 0.0015 ± 0.001, $p = 0.22$, $n = 11$), while for those under ALAN there was a slight increase in nighttime activity across the 8 weeks of exposure (contrast $\pm$SE = 0.00577 ± 0.001, $p = < 0.0001$, $n = 11$; Figure 3A; Figure S3).

In stage 2, we tested whether ALAN exposure had a lasting (post-exposure) effect on individuals, after being returned to dark nights. We found that nighttime activity was elevated for birds that had been previously exposed to ALAN ($n = 11$) compared to birds that had never been exposed ($n = 11$; contrast $\pm$SE = 0.615 ± 0.0571, $p < 0.0001$; Table 1 *bottom;* Figures 2 and 3B).

In stage 3, we tested whether birds would show behavioral habituation to a secondary re-exposure. Indeed, there was a significant effect of previous exposure on responses to re-exposure, indicating habituation (Table 1 *bottom*). ALAN increased nighttime activity in all exposed

Table 1. Effects of ALAN on nighttime activity

Predictor	IRR[a]	SE	CI (95%)	*p*
~ALAN*Day + (1|Bird)				
ALAN	5.98	0.502	5.07, 7.05	**<0.001**
Day	1.00	0.001	1.00, 1.00	0.219
ALAN * Day	1.00	0.002	1.00, 1.01	**0.007**
~ALAN*Previous exposure + Day + (1|Bird)				
ALAN	3.73	0.140	3.46, 4.01	**<0.001**
Day	1.00	0.000	1.00, 1.00	**0.013**
ALAN*Previous exposure				
0 * 1	1.84	0.106	1.65, 2.07	**<0.001**
1 * 1	0.84	0.046	0.76, 0.94	**0.001**

Generalized linear mixed-effect models to analyze behavioral responses (count of active minutes per night) to ALAN exposure. *Top*: Testing for changes in activity over time during chronic ALAN exposure (stage 1, 8 weeks total). *Bottom*: Testing whether nighttime activity depends on previous exposure to ALAN. Effect sizes are reported as Incidence Rate Ratio (IRR). Bold values indicate $p < 0.05$. Repeatability calculated as variance within individuals relative to total variance was 0.7 ± 07 (R ± SE).

[a]IRR = Incidence Rate Ratio.

groups, but less so for birds that had been previously exposed to ALAN (contrast ±SE = −0.172 ± 0.054, $p < 0.008$; Figures 2 and 3B). Repeatability of individual birds, calculated as variance within individuals relative to total variance, was 0.70 ± 07 (R ± SE).

Oxidative stress

There was no difference between antioxidant capacity (oxy; uMol HClO/mL), reactive oxygen metabolites (dROMs; mg H2O2/dL), or our integrative oxidative stress index between groups during acclimation (all p values >0.40). There was, however, a significant effect of sex where males consistently had higher levels of oxidative stress than females (contrast ±SE = 0.789 ± 0.344, adjusted-*p* = 0.034). There was no difference between males and females in oxy ($p = 0.110$, $n = 22$) or dROMs ($p = 0.539$, $n = 22$) individually, meaning sex differences reflect a change in the balance of Oxy and dROMs within individual.

As before, we first compared naive responses in experimental stages 1 and 3 to ensure there were no effects of time (or duration captivity) on oxidative responses to ALAN that may confound later comparisons of first and secondary exposure. We found no effect of ALAN, time, or their interaction on oxidative stress, and no difference between groups exposed to ALAN in stage 1 and stage 3 (Table S2).

In stage 1, we tested whether responses to ALAN depend on duration of exposure by comparing oxidative stress levels after 3 and 8 weeks. There was a significant interaction of duration and ALAN in stage 1 (Table 2 *top*; Figure 3C). For dark-night groups there was an increase in oxidative stress over time (slope = 0.131, se = 0.043, CI = 0.045–0.216, $p < 0.004$). This was driven by an increase in dROMs (slope = 0.240, se = 0.100, CI = 0.039–0.440, $p = 0.02$), with no changes in oxy ($p = 0.27$). For ALAN-exposed groups, there was no change in oxidative stress ($p = 0.50$), and no change in oxy ($p = 0.88$) or dROMs ($p = 0.38$), accordingly. As before, we found that males consistently had higher oxidative stress levels than females (contrast ±SE = 0.61 ± 0.293, adjusted-*p* = 0.05).

In stage 2, we tested whether previous exposure to ALAN had a lasting effect after ALAN was removed. For birds previously exposed to ALAN ($n = 11$), we found an increase in both dROM and oxy absorbance independently, but their combined index of oxidative stress was not different from birds with no previous ALAN exposure ($n = 11$; Table 2 *bottom*; Figure 3D).

In stage 3, we tested whether birds habituate by building resistance to secondary ALAN exposure. Previous exposure to ALAN ($n = 6$) resulted in lower oxidative stress compared to naive birds that were only exposed to ALAN once ($n = 11$), though only with marginal significance ($p = 0.06$; Table 2 *bottom*; Figure 3D). There was no difference in dROM or oxy absorbance independently for birds re-exposed to ALAN (Table 2 *bottom*). Again, males ($n = 11$) had consistently higher oxidative stress than females ($n = 11$; contrast ±SE = 0.735 ± 0.306, adjusted-p< 0.03). Repeatability of oxidative stress for individual birds, calculated as variance within individuals relative to total variance, was 0.23 ± 12 (R ± SE). Repeatability of oxy absorbance and dROM levels were 0.51 ± 0.11 and 0.31 ± 0.12, respectively.

Telomeres

All individual changes in telomere length were negative over time regardless of ALAN treatment, confirming that the time passed between our telomere sampling points was long enough to see some changes in telomere length. Repeatability of telomere length for individual birds, calculated as variance within individuals relative to total variance, was 0.72 ± 0.11 (R ± SE). Males consistently had lower telomere lengths across timepoints (Tukey difference of means = 0.395, $p < 0.001$) and greater rates of telomere attrition (Tukey difference of means = −0.296 $p = 0.07$; Figure S1). There was no effect of ALAN exposure on telomere attrition ($p = 0.44$, $n = 22$; Figure S1).

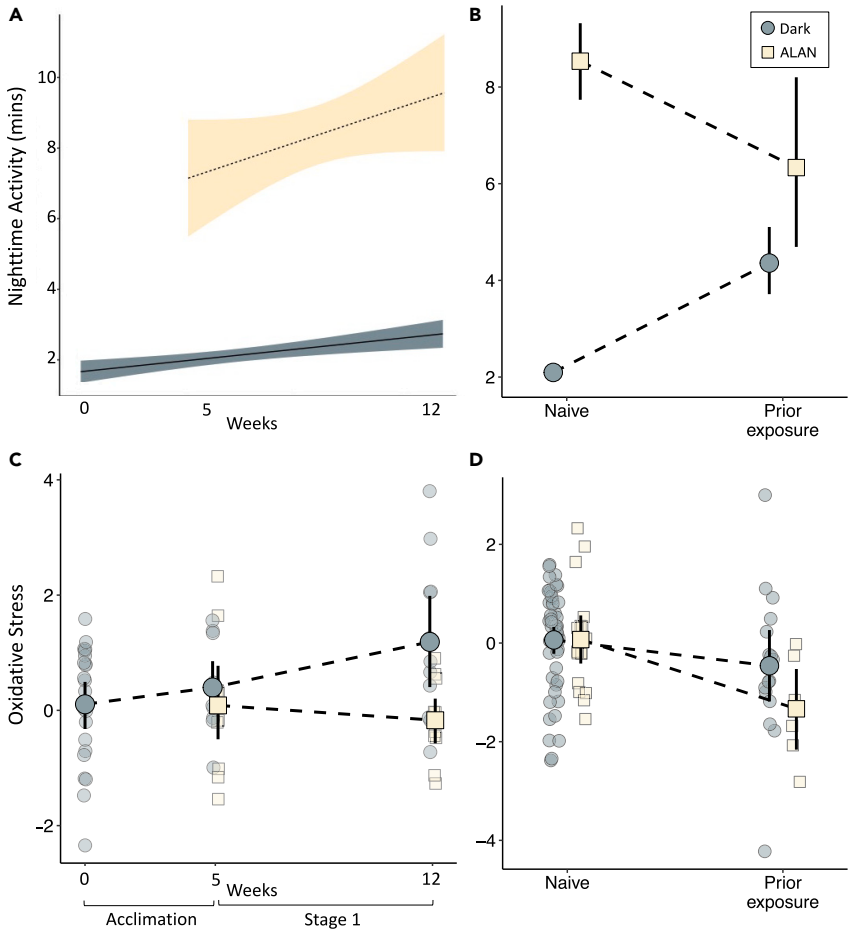

Figure 3. Behavioral and physiological responses to long-term (left) and repeated (right) ALAN exposures

Left: Responses to long-term ALAN exposure, showing acclimation period (weeks 1–4) and stage 1 (weeks 5–12). Gray indicates data collected under dark-night conditions (10L:14D). Yellow represents data collected during ALAN exposure (10L:14dim).

(A) Behavioral responses to ALAN (nighttime activity) increased slightly over time during stage 1 (n = 11), while there was no change in behavior for birds under dark-night conditions (n = 11). Data is shown as linear regression ±standard error.

(C) Oxidative stress increased over time for birds under dark-night conditions during stage 1 (n = 11), but there was no change in ALAN exposed birds (n = 11). Data is shown as mean ± standard error. **Right:** Effects of previous exposure to ALAN. Categories 'Naive' and 'Prior exposure' describe *past* experiences with ALAN (i.e., whether or not birds have been previously been exposed), whereas color coding indicates current treatment (i.e., whether or not birds are currently experiencing ALAN). For example, there is a condition where individuals have had "prior exposure" to ALAN, but are currently under dark-night conditions (also referred to as 'recovery' in discussion). Data is shown as mean ± standard error.

(B) Previous exposure to ALAN had a lasting effect, where nighttime activity was higher post-exposure compared to birds that had never been exposed to ALAN (n = 11). Previously exposed birds reduced behavioral response to ALAN upon secondary re-exposure (n = 6).

(D) Previous exposure to ALAN resulted in lower oxidative stress (n = 11), with the greatest reduction after secondary re-exposure (n = 6).

Gene expression

Only 13 genes were found differentially expressed in our control group (no ALAN) between the beginning and end of the experiment, indicating very little effect of captivity or time, and these genes were not included in analyses of other groups (Figures S4A–S4C). Gene annotation results showing up- and downregulated gene ontology and pathways for all comparisons are reported in Table S3. Individual genes differentially expressed are reported in Tables S4–S6. Volcano plots of pairwise comparisons are provided in supplementary (Figure S4).

First, we compared gene expression after naive ALAN exposure, to the acclimation period (pre-exposure). One hundred and twenty genes were differentially expressed (adjusted $p < 0.05$) with 14 upregulated and 106 downregulated (Figure 4; Table S4).

Next, we analyzed gene expression of birds in recovery compared to naive ALAN exposures, to determine differential expression resulting from previous ALAN exposure. We found 477 genes differentially expressed, with 218 upregulated and 259 downregulated (Figure 4; Table S5). Only 18 genes (3.77%) differentially expressed were the same between recovery and naive exposures, indicating a largely unique response to previous exposure (Figure 4).

Table 2. Effect of ALAN on oxidative stress

	Stress Index				Oxy Absorbance				dROMs			
Predictor	β	SE	CI (95%)	*p*	β	SE	CI (95%)	*p*	β	SE	CI (95%)	*p*
~Sex + ALAN*Day + (1|Bird)												
Sex	0.61	0.293	0.00, 1.20	0.051	−23	17.9	−60, 15	0.222	0.55	0.737	−0.98, 2.1	0.460
Day	0.13	0.042	0.05, 0.22	**0.003**	−2.1	1.88	−5.9, 1.6	0.262	0.24	0.099	0.04, 0.44	**0.019**
ALAN	0.93	0.804	−0.70, 2.50	0.256	−9.3	34.5	−79, 60	0.788	2.00	1.86	−1.8, 5.8	0.291
ALAN * Day	−0.18	0.086	−0.36, −0.01	**0.041**	1.7	3.74	−5.9, 9.2	0.659	−0.40	0.201	−0.80, 0.01	0.055
~Sex + Day + ALAN*Previous exposure + (1|Bird)												
Sex	0.74	0.305	0.10, 1.40	**0.026**	−29	18.7	−68, 9.7	0.133	0.57	0.643	−0.77, 1.9	0.386
Day	−0.03	0.017	−0.07, 0.00	0.060	0.24	0.724	−1.2, 1.7	0.738	−0.08	0.032	−0.14, −0.02	**0.016**
ALAN	0.13	0.297	−0.46, 0.72	0.668	−2.2	12.5	−27, 23	0.860	0.29	0.561	−083, 1.4	0.607
ALAN * Previous exposure												
0 * 1	−0.04	0.395	−0.82, 0.75	0.928	41	17.9	5.2, 76	**0.025**	1.9	0.767	0.41, 3.5	**0.014**
1 * 1	−1.00	0.518	−2.0, 0.04	0.059	30	21.4	−12, 73	0.160	−1.0	0.972	−2.9, 0.94	0.306

Linear mixed-effect models to test oxidative stress response to ALAN. Results of models using a combined stress index as well as individual biomarkers (oxy absorbance and dROMs). *Top:* Test of whether responses to ALAN change across 8 weeks of chronic ALAN exposure (stage 1). *Bottom:* Test of whether response to ALAN depends on previous ALAN exposure (PE). Bold values indicate $p < 0.05$. Repeatability (calculated as variance within individuals relative to total variance) for oxidative stress, oxy absorbance, and dROMs were 0.23, 0.51, and 0.31, respectively.

Finally, we analyzed gene expression of birds re-exposed to ALAN, compared to first-time (naive) exposures, to investigate potential indicators of habituation. We found 395 genes were differentially expressed, with 138 upregulated and 257 downregulated (Figure 4; Table S6). Of these, 90 (22.78%) were similar between re-exposure and recovery groups, indicating some repeatable responses to previous ALAN exposure across groups, regardless of whether individuals were re-exposed (Figure 4). On the contrary, only 2 genes differentially expressed were the same between re-exposure and naive exposures, indicating a largely unique response to secondary exposure compared to naive exposure.

DISCUSSION

Why do some organisms persist in human-altered environments, while others do not? Habituation likely plays an important role in organismal persistence, allowing individuals to respond immediately to novel environmental changes and reduce unnecessary responses to recurring innocuous stimuli.[7] Here, we report the first experimental demonstration of avian habituation to long-term light pollution exposure using dim (0.3 lux) ALAN, to mimic realistic levels of night light birds are exposed to in light-polluted environments. We found evidence of tolerance to chronic (8 weeks) exposure, lasting effects that persist even after 8 weeks of recovery, and reduced responsiveness upon re-exposure, indicating habituation. We also report that, while chronic exposure to ALAN resulted in a modest increase in nocturnal activity, it did not result in any accumulation of oxidative stress, but rather may have enhanced oxidative defenses. Transcriptomic analyses provide some support for these results and gene annotation analyses invite further inquiry into potential effects of chronic ALAN exposure on physiological stress, immune function, and depressive disorders. We present an integrative discussion of behavioral, physiological, and transcriptomic responses to (1) chronic ALAN exposure, (2) previous exposure (after an 8-week period of recovery), and (3) secondary re-exposure, and we discuss how our choice of using dim ALAN (eliciting a very slight change in behavior) may be important for explaining our findings.

Reponses to chronic ALAN exposure

Behavioral responses of naive zebra finches to ALAN in stage 1 were slight (an increase of ~8.5 min of activity per night, on average), but immediate and persistent across 8 weeks. In fact, nighttime activity slightly increased over time, even though daytime activity patterns suggests that perch use tended to decline over time for all groups (Figure 2), which is a typical phenomenon of captive birds.[44] Our findings are novel, as few studies have exposed birds to ALAN for comparable lengths of time. Two other studies exposed birds to 4 weeks of ALAN, with one reporting a persistent behavioral response[45] and the other a slight decline over time.[46] In the present study, and in our previous work,[21,28] we have noticed daily variation in zebra finch activity underlying longer term trends. As such, it could be that the longer-term trend we report here is robust and less likely to have been influenced by random fluctuation in activity than previous work,[46] or that responses vary across species. There was also some individual variation in behavioral responses (here, and in our previous work[28]). While birds on average increased nocturnal behavior over the 8 weeks of exposure, some individual birds decreased responses and others remained constant. Given larger facilities and increased sample sizes, future work looking into the mechanisms underlying this individual variation could be insightful.

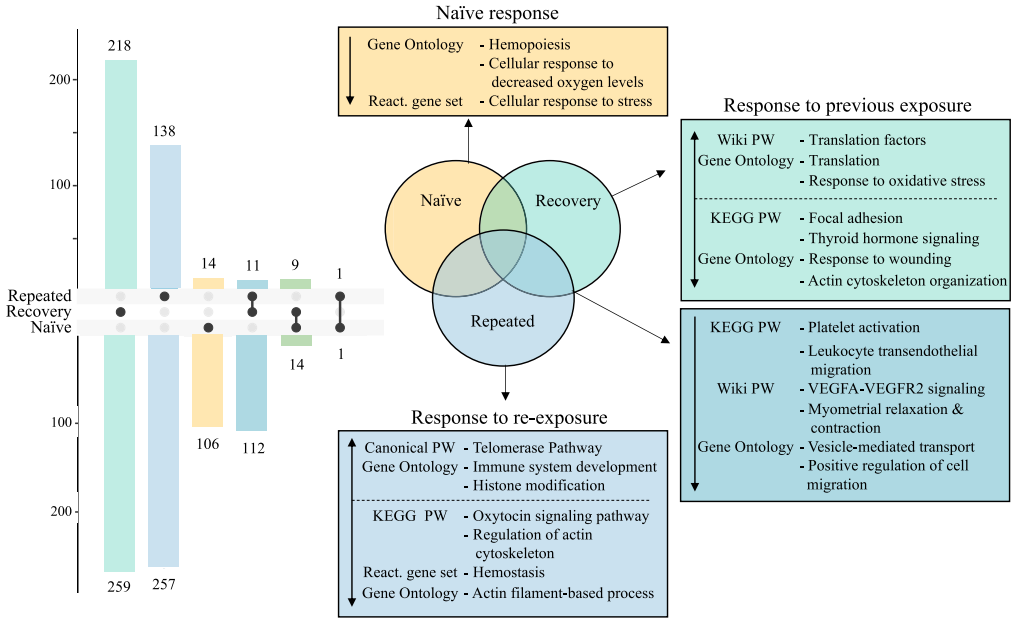

Figure 4. Summary of differential expression and select gene annotation results

'Naive' indicates differential expression after naive ALAN exposure. 'Recovery' indicates differential expression after return to dark-night conditions. 'Repeated' indicates differential expression after secondary re-exposure to ALAN. Upset plot (left) shows the number of genes up- and downregulated per group, with black dots indicating group compared and links between dots indicating genes represented in both groups. Upset plot was created with Intervene[42] and gene annotation analyses was conducted in Metascape.[43] Full gene annotation results reported in Table S3, and gene list reported in Tables S4–S6.

Along with a persistent behavioral response, we found no change in oxidative balance under chronic ALAN exposure. On the contrary, there was a slight increase in dROMs and oxidative stress in birds under dark-night conditions, which was not detected in our ALAN group, suggesting potential protective effects of ALAN. Our blood transcriptome analyses support this, showing that the primary change in individual gene expression during this period was a downregulation of genes related to cellular responses to stress (Table S3; Figure 4). This was surprising, as a large body of literature links sleep debt to oxidative damage. However, one study has demonstrated in rats that small amounts of sleep debt may enhance antioxidant responses.[47] Here, nocturnal activity was consistently elevated under ALAN compared to dark-night conditions, but the effect size is small (~13 min/night of activity on average), which may represent a small enough sleep deficit to result in protective effects instead of pathological effects. Though evidence of this direct relationship between sleep debt and enhanced oxidative response is limited, there are other indirect mechanisms that may also explain our findings. For example, glucocorticoid hormones can activate mechanisms that limit free radical production or upregulate antioxidant defenses, mitigating oxidative damage in the short term.[48] Again, evidence of these protective effects is limited compared to evidence of glucocorticoids increasing oxidative stress.[49] However, we believe this is a plausible explanation because we have previously shown in a similar experiment that 3 weeks of 0.3 lux ALAN exposure does increase glucocorticoid levels in zebra finches.[28] Of course, there are several ways to assess oxidative damage, and though blood serum is the most common tissue used in animal research, oxidative responses may depend on the tissue analyzed.[50] For example, one study found opposite changes in superoxide dismutase (a measure of oxidative protection) during reproduction when comparing blood serum and liver tissue.[51] Additionally, because we measured activity only via use of a perch, we cannot say for sure whether higher activity levels correlate directly with less sleep. It is possible that birds in dark-night conditions were also awake, but engaging in less movement or perch-use.[52] However, in a preliminary study,[53] we used video cameras to quantify a wider range of behaviors (distinguishing between hopping, eating, drinking, and stationary body movement). We found that, while the overall quantity of behaviors was higher under ALAN, birds were engaging in all four behaviors in similar proportions regardless of treatment, rather than shifting the proportion of behaviors more toward 'active' ones (e.g., hopping) under ALAN. Still, linking our results to potential effects of sleep debt it somewhat speculative, as we did not measure sleep here directly.

Responses to previous ALAN exposure

After recovery, previously exposed birds continued to exhibit higher nighttime activity compared to controls with no ALAN exposure. Furthermore, this behavioral response was accompanied by physiological and transcriptomic changes that were unique from the control group and from naive ALAN exposures. To our knowledge, this is the first evidence of a lasting behavioral, physiological, and transcriptomic response to ALAN extending 8 weeks post-exposure.

We found that previous exposure to ALAN elicited an increase in dROMs, but also an appropriate increase in antioxidant defenses (oxy), such that no oxidative stress was detected. Underlying this response, we report upregulation of genes associated with response to oxidative

stress, and downregulation of platelet activation which is otherwise known to induce further ROM production.[54] As we found minimal changes in oxidative stress, it follows that we also found no increase in telomere attrition due to ALAN, which is typically associated with oxidative stress.[55,56] We did find greater telomere attrition in males mirroring their overall higher levels of oxidative stress, showing consistency with these norms, but there was no effect of ALAN treatment. Several other studies have also reported no change in telomere attrition under dim ALAN,[57,58] and our study supports these findings even after substantially longer-term exposure. Still, it is important to note that we only analyzed telomere length at two time points, as red blood cell samples from other timepoints were retained for RNA sequencing, and we cannot rule out the possibility that longer-term ALAN or exposure during critical development stages (rather than adults) may have different effects,[59] or that results may be tissue specific.[56] qPCR techniques are also not as sensitive to very small changes in telomere length, compared to some other methods (e.g., microscopy). However, the fact that we did find differences in TS ratio between sexes, reflecting their respective oxidative balance, and that individual changes in telomere length were negative over time regardless of ALAN treatment, suggest that our protocol and experimental design was able to detect moderate-large variation despite small sample sizes. Taken together, our oxidative balance and telomere measures suggest that despite a lasting effect of previous ALAN exposure on behavior, there was no indication of chronic stress accruement.

On the other hand, transcriptome analyses revealed several other potential underlying effects of previous ALAN exposure (Table S3; Figure 4). First, we found significant downregulation of thyroid hormone (TH) signaling in birds returned to dark nights, which could reflect long-term restricted sleep[60] and/or glucocorticoid elevation,[61] both of which are known to reduce TH signaling. This makes sense, as birds exhibited persistent increases in nocturnal activity even after >2 months returned to dark nights, indicating mild but prolonged sleep disruption. Additionally, we have previously shown that the same type of ALAN exposure increases glucocorticoid levels in zebra finches after 3 weeks.[28] We also found significant downregulation of several immune functions, such as leukocyte transendothelial migration and response to wounding. Though we did not measure other physiological biomarkers of immune function, our results align with previous work showing that dim ALAN exposure (5 lux) can impede cell-mediated immunity[62] and slow wound healing up to 9 days post-exposure in mice.[63] Our results suggest that downregulation of these immunity-related processes can persist, at the transcriptomic level, even after >2 months returned to dark nights.

We also found downregulation of the vascular endothelial growth factor-A (VEGFA) to VEGF receptor 2 (VEGFR2) signaling pathway in birds previously exposed to ALAN. In fact, downregulation of this pathway was found in both recovery and re-exposed groups, indicating a robust response to previous exposure regardless of whether birds were re-exposed. VEGF plays an essential role in blood vessel growth and neurogenesis, and is a reliable biomarker of depressive disorders.[64] Specifically, it has been demonstrated that exposure to various types of stress, including environmental and immune stress, affects expression of VEGF,[65] ultimately leading to the development of depression-like behavior. Our results are supported by one other study showing that just 3 days of ALAN (5 lux) is sufficient to decrease VEGFA expression in the hippocampus and cause depressive behavior in mice.[66] Here, we report that downregulation of VEGFA signaling can persist 11 weeks after ALAN exposure. Because our data reflect only gene expression in red blood cells, more targeted research would be needed to determine whether these changes represent precursors to pathology or depression. Nonetheless, a substantial body of biomedical research links ALAN exposure to depressive disorders,[67,68] due to the prevalence of depression in night shift workers,[69] presumably as a result of prolonged melatonin suppression.[70] Our findings (and those of[66]) support that VEGF signaling may be an alternative link between ALAN exposure and depressive disorders and warrant more research in this direction.

Responses to re-exposure

We found that previous exposure to ALAN led to an attenuated behavioral response upon re-exposure and no oxidative damage, supporting our predictions for habituation. Measuring changes in response across multiple exposures is a classic way to disentangle true habituation from chronic continuous exposure and has been demonstrated in other systems.[71,72] However, in habituation studies, interstimulus intervals are rarely greater than a couple of days, and responses typically return to normal with increasing lengths of interstimulus intervals.[73] Here, we demonstrate substantial long-term 'memory' of previous ALAN exposure with reduced responsiveness to re-exposure even 2 months later. Additionally, we report lower levels of oxidative stress in birds re-exposed to ALAN compared to those only exposed once (though marginally significant). Taken together, our data supports the idea that mild sleep debt caused by dim ALAN exposure may contribute to resilience against oxidative damage. However, it is important to note that the effect of dim ALAN on nighttime activity was small; birds were 25.8% less active during re-exposure than initial exposure but this translates to a difference of only 2.2 min. Still, we consider our findings notable given the length of our period of data collection and interstimulus period. Raw activity data (Figure S2) has been provided for an additional visual of the variation in response between previously exposed and naive individuals.

Transcriptome analyses revealed that responses to re-exposure varied substantially from naive exposures, with only 2 differentially expressed genes overlapping. In contrast, 22.78% of genes overlapped between birds re-exposed and those in recovery, which we interpret as underlying a persistent response to previous ALAN exposure, regardless of whether birds were re-exposed. Still, many transcriptomic responses emerged that were unique to re-exposure (Figure 4). For example, in our re-exposed group we found significant upregulation of immune system development and downregulation of oxytocin signaling (Table S3). Oxytocin is primarily known for its involvement in reproduction and social behaviors,[74] but can also modulate the glucocorticoid stress response and reduce stress-related disease, anxiety, and depression.[74,75] Oxytocin is also an antioxidant and anti-inflammatory,[76,77] and participates in a suite of immune responses.[78] It is well documented that ALAN exposure can affect glucocorticoids, depression, anxiety, inflammation, and relevant immune functions, but no studies have specifically linked ALAN to down-regulation of oxytocin. Future work should investigate the mechanisms by which ALAN induces some of these pathologies and the potential role of oxytocin signaling.

We also found a significant upregulation in the telomerase pathway in re-exposed birds. Telomerase is responsible for increasing telomere length,[79] which could have contributed to the lack of telomere attrition reported in our study. However, downregulation of telomerase was only observed in birds after re-exposure to ALAN (end of stage 3), and we do not have telomere measurements at this time point. Additionally, while telomerase activity may be beneficial in reducing effects of aging, it is also an aspect of uncontrolled cell growth and tumor formation,[80] and most (90%) of cancers involve overactive telomerase activity.[81] Indeed, as a short-lived species, telomerase expression is known to be low in blood of healthy adult zebra finches, only increasing toward the end of life in specific aging tissues (e.g., muscle, liver, gonads), where it is suggested to play a role in cancer development. As ALAN exposure has also been shown to increase the risk of cancer in humans,[82] future work should consider the potential role of increased telomerase activity.

Dim ALAN

Our choice of using dim (0.3 lux) ALAN is likely important for interpreting our findings. We use 0.3 lux ALAN to mimic realistic light levels birds are exposed to in light polluted environments. Previous work has shown that increases in nighttime activity in zebra finches under similarly dim ALAN is a masking effect, where endogenous circadian rhythms are not altered, despite changes to behavior.[27,28] This finding is so far unique to studies focusing on dim ALAN, compared to studies that use brighter intensities (e.g., 5 lux).[22,23] Masking, as a strategy to flexibly adjust to natural moonlight fluctuations,[83,84] may therefore enable birds to tolerate to light pollution at these dim levels, and persist in light polluted environments. Dim ALAN may also be more effective in leading to habituation than brighter ALAN.[7] Other experimental studies on habitation to anthropogenic disturbance suggest the strength of a stimulus is generally important in developing tolerance rather than sensitization.[7] Additionally, several studies have demonstrated that behavioral responses of birds to ALAN show a clear dose-dependent relationship.[27,45] Indeed, the dim levels of ALAN we use in this study had very small (though persistent) effects on behavior overall, and this aligns with other dose-dependent studies. We propose that the capacity to habituate is likely to depend on light intensity,[7] as bright ALAN is known to elicit different responses and cause severe health consequences.[14,17] Our results suggest that reducing ALAN intensity by minimizing excessive or redundant lights may be an effective strategy for promoting habituation and mitigating negative consequences of light pollution for resident birds.

Just as the responses to dim ALAN treatment may reflect an unnaturally prolonged adaptive response to full-moon conditions, the lack of activity under dark-night conditions may represent an unnaturally prolonged response to new moon and cloudy conditions. In a truly natural setting animals regularly experience light at night from partial moonlight and stars in the range of 0.001–0.01 lux, and activity does sometimes vary according to naturally fluctuating light levels. Our light meter was not sensitive enough to detect potential light levels below 0.01 lux. So, we cannot rule out the possibility that our dark-night conditions were, in fact, unnaturally dark, and this resulted in artificially lower levels of activity than would have been observed under "natural" conditions. Following this, it could also be the case that unnaturally dark conditions induced some stress, alternatively explaining our finding that oxidative stress levels increased slightly over time under dark-night conditions. Indeed, one study found that great tits (Parus major) actually prefer dimly lit roosting sites to dark ones.[85] We suggest follow up research use higher sensitivity light meters to rule out these possibilities.

Limitations of the study

Though ALAN is known to disrupt many behaviors and physiological processes, many animals persist in light polluted environments, perhaps because of habituation. We have experimentally demonstrated that zebra finches have the potential for long-term habituation to ALAN, even after an interstimulus interval of 8 weeks. We found that zebra finches can behaviorally habituate to dim ALAN, reducing their behavioral responses to re-exposure, without incurring oxidative damage or accelerated telomere attrition. Still, transcriptome analyses suggest there may be underlying molecular responses that deserve further investigation, exemplifying the importance of linking whole-animal responses to environmental change across biological scales. Our transcriptomic data represent a starting point for understanding the complex genetic responses to ALAN in diurnal birds, as more research is needed to understand the potential long-term negative consequences of ALAN exposure on the many genes and pathways we report here (Tables S3, S4–S6), which we have not the space to discuss. And, while focusing on the blood transcriptome created a unique opportunity to document changes within individuals over time, the effects we were able to detect are likely limited, compared to what might be found in analysis of other critical tissues.[86] Gene expression, oxidative responses, and telomere attrition can all vary across tissues.[51,86,87] Finally, the ability for birds to habituate to ALAN may vary across species, depending on life history strategy, migratory status, or photosensitivity.[88] The capacity for free-living populations to habituate may also differ from captive birds, as light pollution is almost always accompanied by other anthropogenic disturbances such as noise, air, and water pollution, which may have synergistic effects.[89] Nonetheless, our findings emphasize the importance of considering how responses to anthropogenic disturbance may change in populations over time in urban management decisions, rather than overgeneralizing results of short-term studies.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Conceptualization, V.J.A., Y.Z., and J.Q.O.; Methodology, S.M.F., V.J.A., C.H., J.L., and W.C.Y.; Investigation, V.J.A., C.H., S.M.F., J.L.; Writing – Original Draft, V.J.A; Writing – Review and Editing, J.L., S.M.F, Y.Z., and J.Q.O.; Funding Acquisition, Y.Z. and J.Q.O.; Resources, W.C.Y. and J.Q.O.; Supervision, V.J.A., Y.Z., W.C.Y., and J.Q.O.

DECLARATION OF INTERESTS

Authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Diacron Oxy Absorbance	Diacron International	MC435
Diacron dROM	Diacron International	MC003
DNeasy Blood & Tissue kit	Qiagen	#69504
Deposited data		
RNA sequence data	NCBI SRA	accession number PRJNA977995
Zebra Finch activity data	Dryad	https://doi.org/10.5061/dryad.rjdfn2zhs
Zebra Finch oxidative stress data	Dryad	https://doi.org/10.5061/dryad.rjdfn2zhs
Zebra Finch telomere data	Dryad	https://doi.org/10.5061/dryad.rjdfn2zhs

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Valentina Alaasam (valentina.alaasam@gmail.com)

Materials availability

This study did not generate new unique reagents.

Data and code availability

- RNA-seq data have been deposited at NCBI SRA Database and available at accession number PRJNA977995 or at the following link: https://www.ncbi.nlm.nih.gov/sra/PRJNA977995
- All other data generated from this research has been made available on Dryad at https://doi.org/10.5061/dryad.rjdfn2zhs
- This paper does not report original code
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Twenty-two adult zebra finches (11 male, 11 female) were housed at the University of Nevada, Reno in individual cages with access to food (Finch Mix Fancy, Volkman Seed Factory, Ceres, California, USA) and water *ad libitum*. All birds were between 1-2 years old, though the exact hatch date of individuals is unknown. All cages were housed in a single facility, arranged in a 4x6 grid, with the assignment of treatment groups spatially randomized. Each cage was installed with its own independent day- and night-light. Light occlusion shades were installed around each cage to block vision and prevent nightlights from affecting neighbors. White noise was played constantly to disrupt inter-individual communication and sound. Each cage also included an active perch programmed to record activity (elaborated below). Treatment group allocation was randomized using the randomer number generator in excel. Treatment group was blind to the observers during all sampling, blood processing, and analyses. Because both males and females were included in our study, sex was included as a fixed effect in all initial models (see quantification and statistical analysis below). All procedures were conducted in accordance with National Institute of Health Ethical Use of Animals guidelines and were approved by the University of Nevada, Reno Institutional Animal Care and Use Committee.

METHOD DETAILS

Experimental design

Birds were acclimated to a normal photoperiod of 10L:14D for four weeks. Day lights turned on at 0700 and off at 1700. Day lights were provided by a 1.4-Watt LED rated at 95 Lumens at a color temperature of 5000K. For birds under ALAN treatment, night lights turned on at 1659 and off at 0701 so that the transition from day to night was seamless. Night lights were constructed using commercial LED strip lighting rated at 5000K. Illuminance of night-lights was standardized at 0.3 lux using an Extech Easyview Digital Light Meter (model EA13). We use 0.3 lux ALAN to mimic light levels birds are exposed to in light polluted environments.[90] To standardize nighttime illuminance of each cage, measurements were taken at perch height in the center of the cage and at opposite corners (farthest locations away from light) and then averaged to 0.3lx $\pm$ 0.01. For control birds, a night-light was installed but not plugged into power, and nighttime illumination was $<$ 0.01 lux.

The duration of the experiment was 23 weeks (Figure 1). For the first 4 weeks, all birds were kept in standard 10L:14D conditions to acclimate to laboratory conditions. Experimental stages 1 (weeks 5-12) and 3 (weeks 21-23) involved ALAN exposure for some groups (see below). In stage 2, between ALAN exposures (weeks 13-20), all birds were returned to acclimation conditions (Figure 1), which we call the 'recovery' stage. In stage 1, all exposed birds were naïve to ALAN, and we tested whether naïve responses to ALAN depend on duration of exposure, documenting response continuously across 8 weeks of L:Ldim conditions. In stage 2 (recovery), we tested whether ALAN exposure has a lasting (post-exposure) effect on individuals, after being returned to dark nights. In stage 3, we tested whether the experimental group show behavioral habituation upon a secondary re-exposure.

Birds were randomly divided into four groups, while keeping sexes evenly distributed. We kept one control group in acclimation conditions for the remainder of the experiment and never exposed to ALAN, to control for potential effects of time (*i.e.*, duration of captivity or time of year). Two groups were each exposed to dim ALAN (L:Ldim) in either stage 1 or stage 3 (hereafter, "single-exposure (early)", and "single-exposure (late)", Figure 1), to control for variation in response to ALAN over time (i.e. an interaction between ALAN exposure and duration of captivity). Our experimental group was exposed to ALAN during both stages 1 and 3 ("repeated", Figure 1) to test for effects of re-exposure.

Blood samples were collected five times throughout the experiment. Pre-exposure blood samples were collected at the end of the acclimation period (week 4). To test for effects of first-time (naïve) exposure to ALAN and long-term duration of exposure on oxidative stress, blood samples were collected in stage 1 at the end of weeks 7 and 12. To test for lasting (post-exposure) effects of ALAN, blood samples were taken at the end of stage 2 (week 20). To test for effects of re-exposure, blood samples were collected at the end of stage 3 (week 23). All samples were collected between 9:00-9:15am. Blood was collected from the brachial vein using a heparinized capillary tube within 10 minutes of capture. Blood was immediately centrifuged for 10 minutes to separate red blood cells and plasma and then stored at -80F until bioassays.

Behavior

Active perches in each cage were designed to record hops on/off the perch constantly, which is a reliable measure of avian activity.[21,91] Briefly, perches were connected to an optical end-stop so that the downward force of the bird caused a wooden perch to block the signal between the emitter and the receiver on the end-stop, sending a 1 or 0 to a computer every 0.23 seconds depending on whether the bird was on or off the active perch, respectively. Data was captured on a Dell Precision 5810 Tower equipped with an Intel Xeon Processor (E5-1620 v3) running at 3.5GHz, and an AMD FirePro W4100 graphics card. Activity for each individual was recorded as a binomial 1 (active) or 0 (non-active) every minute and then summed, resulting in the total number of minutes active per day and night as a metric of locomotor activity. Though multiple hops may have been recoded within 1 minute, we chose to record activity as a binomial per minute (rather than raw # of hops) to reduce error associated with perch sensitivity, resulting in a count of minutes active per night rather than hop frequency. Details on perch manufacturing and programming follows Alaasam et al., 2018.[28]

Oxidative stress

Oxidative stress was measured in plasma from all blood sampling events (Figure 1). Derivatives of reactive oxygen metabolites (dROMs) were measured using a spectrophotometric assay kit (Diacron, Grosseto, Italy) that detects the level of hydroperoxides, which signal both protein and lipid oxidative damage.[92] Antioxidant capacity (oxy) was measured using an oxy-absorbance assay kit (Diacron, Grosseto, Italy), which measures blood antioxidant barrier by quantifying the oxidant action of hypochlorous acid (HClO). We calculated the oxidative stress index, which represents the difference between the standardized dROM and Oxy values for each individual, such that higher values of our index correspond to a greater differential between dROM and Oxy.[93]

For dROMs, we diluted 5µl of blood plasma into 100µl of the provided acidic buffered solution following an end-point protocol according to manufacturer's instructions. For Oxy, we diluted 2µl of blood plasma into 100µl of distilled water and 100µl of HClO solution following manufacturer's protocol. All samples were run in triplicate. Inter-assay variation was calculated using six replicate standards as the standard deviation of the standards divided by the average. Because a biologically relevant assessment of oxidative stress includes both metrics, we used an integrative index of oxidative stress, incorporating both levels of dROMs and the absorbance capacity of the blood (Oxy) within individuals. We standardized dROM and Oxy using the formula $xs = (x - m) / sd$), where xs represents a standardized value, x represents the corresponding original value, and *m* and sd represent the mean and standard deviations, respectively.

Telomeres

Telomere length was measured from red blood cells collected at weeks 7 and 20 (Figure 1). DNA was extracted from RBCs using the DNeasy Blood & Tissue kit (Qiagen #69504) following manufacturer instructions. Telomere lengths were quantified via qPCR with protocols adapted from published methods,[94] and recommendations for design and reporting.[95] In brief, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the reference gene. We amplified telomeres using the telc telg primer set (telg: 5'-ACAC TAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3'; telc: 5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3') and amplified GAPDH using the GAPDH-F and GAPDH-R primer set (GAPDH-F: 5'-AACCAGCAAAGTACGATGACAT-3'; GAPDH-R: 5'-CCAT CAGCAGCAGCCTTCA-3').[96,97] Prior to analysis we diluted DNA samples to a concentration of 3.33 ng/uL using ultrapure water. Analyses were conducted on a single 384-well plate, running each sample in triplicate for each primer set, with timepoints and treatment groups randomly distributed. Each reaction consisted of a total volume of 10 uL containing 5 uL GoTaq qPCR Master Mix (Promega #A6001), 200 nM each telg/telc or GAPDH-F/GAPDH-R, and 1 uL of diluted DNA (3.33 ng DNA in total). Reactions were conducted according to GoTaq

specifications (2 min at 95°C, followed by 40 cycles of 15 sec at 95ºC and 1 min at 60°C) using a QuantStudio 5 Real-Time PCR system (Applied Biosystems). GoTaq Master Mix shows high specificity in zebra finches.[98] Telomere lengths were calculated by the $2^{\Delta\Delta Ct}$ method wherein the T/S ratio for each sample is calculated by the formula where $\Delta\Delta Ct = (Ct^{telomere} - Ct^{GAPDH})_{reference} - (Ct^{telomere} - Ct^{GAPDH})_{focal}$. All samples were compared to a pooled reference standard. All samples fell within the bounds of the standard curve for each primer set and efficiencies were 95.8% (telomere) and 110.8% (GAPDH). We did not include replicates of samples to report the intra-class correlation coefficients; however intraplate coefficients of variation were 0.94% and 0.67% for telomeres and GAPDH, respectively. The standard error deviation of technical replicates was 0.126 for GAPDH and 0.454 for telomeres.[99] Water controls run without DNA template showed no background amplification.

RNA-Sequencing

Transcriptome analyses were conducted using red blood cells from weeks 4 (acclimation), 12 (end of stage 1), and 23 (end of stage 3; Figure 1). Samples were mixed in TRIzol with a pestle for 2 minutes, then inverted for 10 minutes at room temperature. 200ul of chloroform was added to each sample, inverted, and then centrifuged at 4°C for 20 minutes. Supernatant was removed and samples were placed in 500ul of isopropanol overnight at -20°C. The next day samples were centrifuged at 4°C for 20 minutes, the supernatant was removed, and the remaining pellet was washed with ethanol twice. The pellet was left to dry for 5 minutes and then 20ul of nuclease-free water was added and left to sit for 30 minutes. Samples were analyzed on a NanoDrop 1000 (Thermo Scientific) to measure concentration and quality. Library prep and sequencing was conducted by Novogene Corporation. Library prep was done using a NEBNext Ultra II RNA Library Prep kit by Illumina. Briefly, messenger RNA was purified using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers followed by the second strand cDNA synthesis. End repair, A-tailing, adapter ligation, size selection, amplification, and purification was then conducted before sequencing on an Illumina NovaSeq 6000 S4 flowcell.

QUANTIFICATION AND STATISTICAL ANALYSIS

All models were run in R version 4.1.1 and linear models were conducted using the package lme4.[100] Repeatability of random effects (always individual bird) was calculated using the package the package rptR.[101] For behavioral measurements, we used a generalized linear mixed model with individual as a random effect to account for repeated measures of individuals across time. All initial models included sex (factor) and an interaction of ALAN (factor) and duration of captivity (integer) as fixed effects and activity (minutes active per night) as the response. Post-hoc tests for pair-wise comparisons were run using the package emmeans version 1.7.1-1,[102] with a Bonferroni correction for multiple comparisons. Models were checked for over-dispersion and zero-inflation using the package DHARMa[103] and fit with a Poisson distribution. Effect sizes are reported as Incidence Rate Ratios where a value of 1 represents no difference between the groups, and deviations from one indicate positive (>1) and negative (<1) associations.

For oxidative stress measurements we used linear mixed models where all models included sex and an interaction of ALAN and duration of captivity as fixed effects, and individual as a random effect. Identical models were run using the integrative index of oxidative stress and raw values of oxy and dROMs as response variables, to determine whether changes in oxidative stress (a combined metric) were driven by an increase in dROM or a decrease in absorbance capacity. Models met assumptions of a normal distribution and were fit with a Gaussian distribution.

For telomere measurements we used linear mixed models with sex and an interaction of ALAN and time as fixed effects, and individual as a random effect. We also ran a linear model using the difference in telomere length within individuals over time as a response variable, and ALAN treatment as a fixed effect. Models met assumptions of normal distribution and were fit with a Gaussian distribution.

For transcriptome analysis, sequencing depth ranged between 17.6 and 34.7 million mate-pairs over all samples. Adaptor sequences and low-quality bases were trimmed from the raw sequencing data using Trim Galore[104] and quality control analysis was conducted using FastQC. Trimmed reads were aligned to the zebra finch Vertebrate Genomes Project reference assembly (NCBI: GCF_003957565.2) using STAR v2.7.10a[105] achieving a mean alignment rate of 92.5%. Gene-wise expected read counts were computed over all 21,732 reference genes using RSEM[106] and differential gene expression analysis was conducted using DESeq2.[107] For differential expression analysis, samples were placed into four exposure groups, including no exposure, one exposure, two exposures, and recovery. To control for high variability between individuals, an additive linear model was used to analyze the effect of exposure group on gene expression while blocking for the individual. Differentially expressed genes with a false discovery rate (FDR) less than 0.05 and $\log_2$(fold-change) > 1 were identified from pairwise comparisons between exposure groups using the Wald test. All gene ontology analyses were conducted with Metascape,[43] using a q-value cutoff of 0.1.

To confirm the results were not due to chance alone, we compared the number of differentially expressed genes (DEGs) in each contrast to the mean number of DEGs found in 1,000 randomized trials. In each trial, the influence of ALAN treatments was removed by randomly assigning the treatments within an individual, keeping the original samples intact. DEGs were computed from each random dataset using the DESeq2 workflow described above and the total number of DEGs identified in the original dataset was compared to the binomial sampling distribution produced by the 1,000 random trials ($p<0.05$). All contrasts yielded a p-value close to zero, indicating a significant difference between the reported DEGs and those identified when treatment effects were removed.

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Supplemental information

What happens when the lights are left on? Transcriptomic and phenotypic habituation to light pollution

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Supplementary materials for "*What happens when the lights are left on? Transcriptomic and phenotypic habituation to light pollution*"

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Table S1: Generalized linear mixed-effect model to test whether behavioral responses of naïve birds to ALAN (count of active minutes per night) depend on duration of activity / time of year animals were exposed (stage). There was no significant effect of sex, so it was dropped from the final model. Fixed effects include day (integer) and the interaction of ALAN (factor) and stage (factor), with individual as a random effect to account for repeated measures and a Poisson distribution.

Predictor	**IRR**[1]	**SE**	**CI(95%)**	***p***
Day	1.00	0.002	1.00, 1.00	0.433
ALAN	2.31	0.919	1.06, 5.04	**0.035**
ALAN * stage				
0 * 3	0.87	0.145	0.63, 1.21	0.407
1 * 3	1.27	0.539	0.55, 2.92	0.579

[1] IRR = Incidence Rate Ratio

Table S2: Linear mixed-effect models to test whether oxidative responses of naïve birds to ALAN depend on duration of captivity or time of year animals were exposed. Fixed effects include sex (factor), ALAN treatment group (factor) and day (integer), with individual bird as a random effect to account for repeated measures. There was no significant interaction of ALAN and day, so the interactive term was dropped from the final model. Results of models using a combined stress index as well as individual biomarkers (oxy absorbance and dROMs).

	Stress Index				Oxy Absorbance				dROMs			
Predictor	β	SE	CI (95%)	*p*	β	SE	CI (95%)	*p*	β	SE	CI (95%)	*p*
Sex	0.79	0.342	0.07, 1.5	**0.033**	-33	19.6	-73, 8.1	0.110	0.51	0.815	-1.2, 2.2	0.539
Day	-0.02	0.017	-0.06, 0.01	0.166	0.19	0.635	-1.1, 1.5	0.768	-0.06	0.039	-0.14, 0.02	0.134
ALAN	-0.09	0.288	-0.67, 0.48	0.748	-2.0	10.7	-24, 19	0.850	-0.25	0.657	-1.6, 1.1	0.704

Table S3. Gene annotation analysis of down- and upregulated genes for 1) naïve ALAN exposure compared to acclimation period (pre-ALAN); 2) Recovery 11 weeks post-exposure compared to during naïve exposures, and 3) Secondary re-exposure compared to naïve exposure. Gene annotation analysis conducted in Metascape [4]. Only top 20 results are shown, and all *q*-values of less than 0.05 are bolded. Full output available on GitHub (https://github.com/valaasam)

	Direction	Category	Name	# of Genes	*p*	*q*
Naïve Exposure	Down-regulated	Reactome Gene Sets	Cellular response to stress	15	< 0.001	**< 0.001**
			Neddylation	8	< 0.001	**0.004**
			Golgi Associated Vesicle Biogenesis	4	< 0.001	**0.044**
			Oxygen-dependent proline hydroxylation of Hypoxia-inducible Factor Alpha	4	< 0.001	0.074
		GO Biological Processes	Hemopoiesis	11	< 0.001	**0.014**
			Response to peptide	8	< 0.001	0.089
			Cellular response to decreased oxygen levels	5	< 0.001	0.089
Recovery	Down-regulated	KEGG Pathway	Platelet activation	19	< 0.001	**< 0.001**
			Focal adhesion	19	< 0.001	**< 0.001**
			Leukocyte transendothelial migration	11	< 0.001	**< 0.001**
			Thyroid hormone signaling	11	< 0.001	**< 0.001**
		WikiPathways	VEGFA-VEGFR2 signaling	22	< 0.001	**< 0.001**
			Myometrial relaxation and contraction	11	< 0.001	**< 0.001**
		Canonical Pathways	PID FAK	9	< 0.001	**< 0.001**
		Reactome Gene Sets	Muscle contraction	14	< 0.001	**< 0.001**
		GO Biological Processes	Response to wounding	29	< 0.001	**< 0.001**
			Actin cytoskeleton organization	28	< 0.001	**< 0.001**
			Positive regulation of cell migration	24	< 0.001	**< 0.001**
			Regulation of vesicle-mediated transport	23	< 0.001	**< 0.001**
			Cellular response to nitrogen compound	23	< 0.001	**< 0.001**
			Regulation of kinase activity	23	< 0.001	**< 0.001**
			Circulatory system process	21	< 0.001	**< 0.001**
			Cellular response to organic cyclic compound	19	< 0.001	**< 0.001**
			Regulation of cell-substrate junction organization	10	< 0.001	**< 0.001**
			Actomyosin structure organization	10	< 0.001	**< 0.001**
			Release of sequestered calcium ion into cytosol	8	< 0.001	**< 0.001**
			Cell-substrate junction assembly	7	< 0.001	**< 0.001**
	Up-regulated	WikiPathways	Translation factors	6	< 0.001	**< 0.001**
		Reactome Gene Sets	Asparagine N-linked glycosylation	9	< 0.001	**0.023**
			Hypusine synthesis from eIF5A-lysine	3	< 0.001	**< 0.001**
		GO Biological Processes	Translation	33	< 0.001	**< 0.001**
			Endomembrane system organization	17	< 0.001	**< 0.001**
			Protein catabolic process	16	< 0.001	**0.004**
			Ribosome biogenesis	13	< 0.001	**< 0.001**
			Regulation of cellular amide metabolic process	12	< 0.001	**0.033**
			Positive regulation of organelle organization	11	< 0.001	0.058
			Aerobic electron transport chain	10	< 0.001	**< 0.001**
			Cellular component disassembly	10	< 0.001	**0.006**

			Response to oxidative stress	9	< 0.001	0.074
			Mitochondrial gene expression	8	< 0.001	**0.001**
			Regulation of proteolysis involved in protein catabolic process	8	< 0.001	**0.017**
			Protein localization to organelle	7	< 0.001	**0.001**
			Vacuole organization	7	< 0.001	**0.022**
			Protein folding	7	< 0.001	0.068
			Regulation of ubiquitin protein ligase activity	4	< 0.001	**0.003**
			tRNA threonylcarbamoyladenosine metabolic process	3	< 0.001	**0.033**
		CORUM	Parvulin-associated pre-rRNP complex	3	< 0.001	**0.009**
Re-exposure	Down-regulated	KEGG Pathway	Regulation of actin cytoskeleton	21	< 0.001	**< 0.001**
			Platelet activation	17	< 0.001	**< 0.001**
			Leukocyte transendothelial migration	14	< 0.001	**< 0.001**
			Oxytocin signaling pathway	14	< 0.001	**< 0.001**
		WikiPathways	VEGFA-VEGFR2 signaling pathway	22	< 0.001	**< 0.001**
			Myometrial relaxation and contraction pathways	16	< 0.001	**< 0.001**
		Reactome Gene Sets	Hemostasis	35	< 0.001	**< 0.001**
			GPCR downstream signaling	25	< 0.001	**< 0.001**
			Cell-Cell communication	13	< 0.001	**< 0.001**
			Smooth Muscle Contraction	8	< 0.001	**< 0.001**
		GO Biological Processes	Actin filament-based process	35	< 0.001	**< 0.001**
			Regulation of body fluid levels	24	< 0.001	**< 0.001**
			Regulation of vesicle-mediated transport	24	< 0.001	**< 0.001**
			Positive regulation of cell migration	24	< 0.001	**< 0.001**
			Cell morphogenesis	23	< 0.001	**< 0.001**
			Regulation of anatomical structure size	21	< 0.001	**< 0.001**
			Regulation of GTPase activity	21	< 0.001	**< 0.001**
			Regulation of small GTPase mediated signal transduction	19	< 0.001	**< 0.001**
			Regulation of cell morphogenesis	17	< 0.001	**< 0.001**
			Small GTPase mediated signal transduction	15	< 0.001	**< 0.001**
	Up-regulated	Canonical Pathways	PID Telomerase Pathway	5	< 0.001	**0.012**
		GO Biological Processes	Immune system development	15	< 0.001	**0.003**
			Histone modification	11	< 0.001	**0.003**
			Neural tube closure	6	< 0.001	**0.006**
			Chaperone cofactor-dependent protein Refolding	4	< 0.001	**0.012**
			Negative regulation of macroautophagy	4	< 0.001	**0.016**
			Nucleocytoplasmic transport	7	< 0.001	**0.046**
			Heterochromatin formation	4	< 0.001	0.052
			Protein autophosphorylation	6	< 0.001	0.071
			Protein hydroxylation	3	< 0.001	0.091
			Nucleus localization	3	< 0.001	0.091
			Regulation of cell cycle process	11	< 0.001	0.100
			Regulation of dephosphorylation	5	< 0.001	0.100

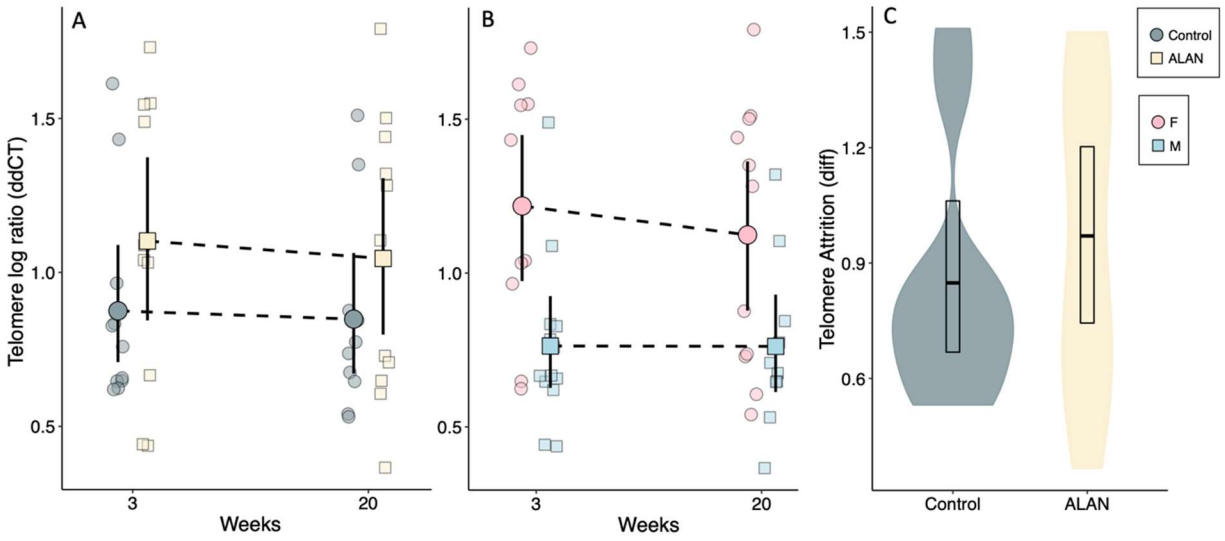

Figure S1. Telomere lengths did not differ between ALAN and control groups at either timepoint (A). Males consistently had shorter telomeres that females across timepoints (B). Individual changes in telomere length over time were all positive, indicating a shortening of telomeres over time, but there was no difference in degree of attrition between groups (C).

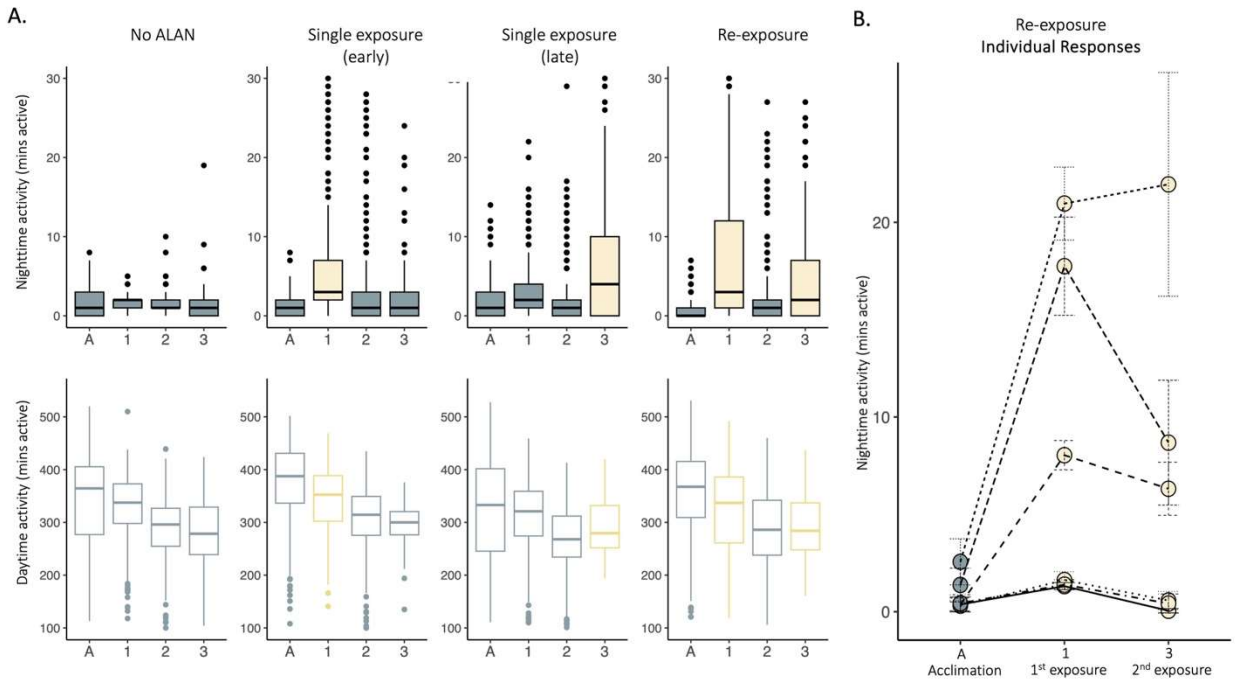

Figure S2. Effect of ALAN on activity, shown for treatment groups. **(A).** Boxplot version of Figure 2 in main text, showing raw data. Nighttime (top) and daytime (bottom) activity, measured as total minutes active in the night and/or day, across stages of the experiment. Stage "A" represents acclimation period. Note differences in y axis between daytime and nighttime plots. **(B).** A closer look at individual responses to repeated ALAN exposure. Here we show individual birds (n=6) in our experimental group that were exposed to ALAN in both stages 1 and 3. Points represent individual averages (across days of exposure), and error bars show standard errors. Stage 2 here was omitted, to illustrate the change in magnitude of response between first and second exposure (*i.e.*, habituation).

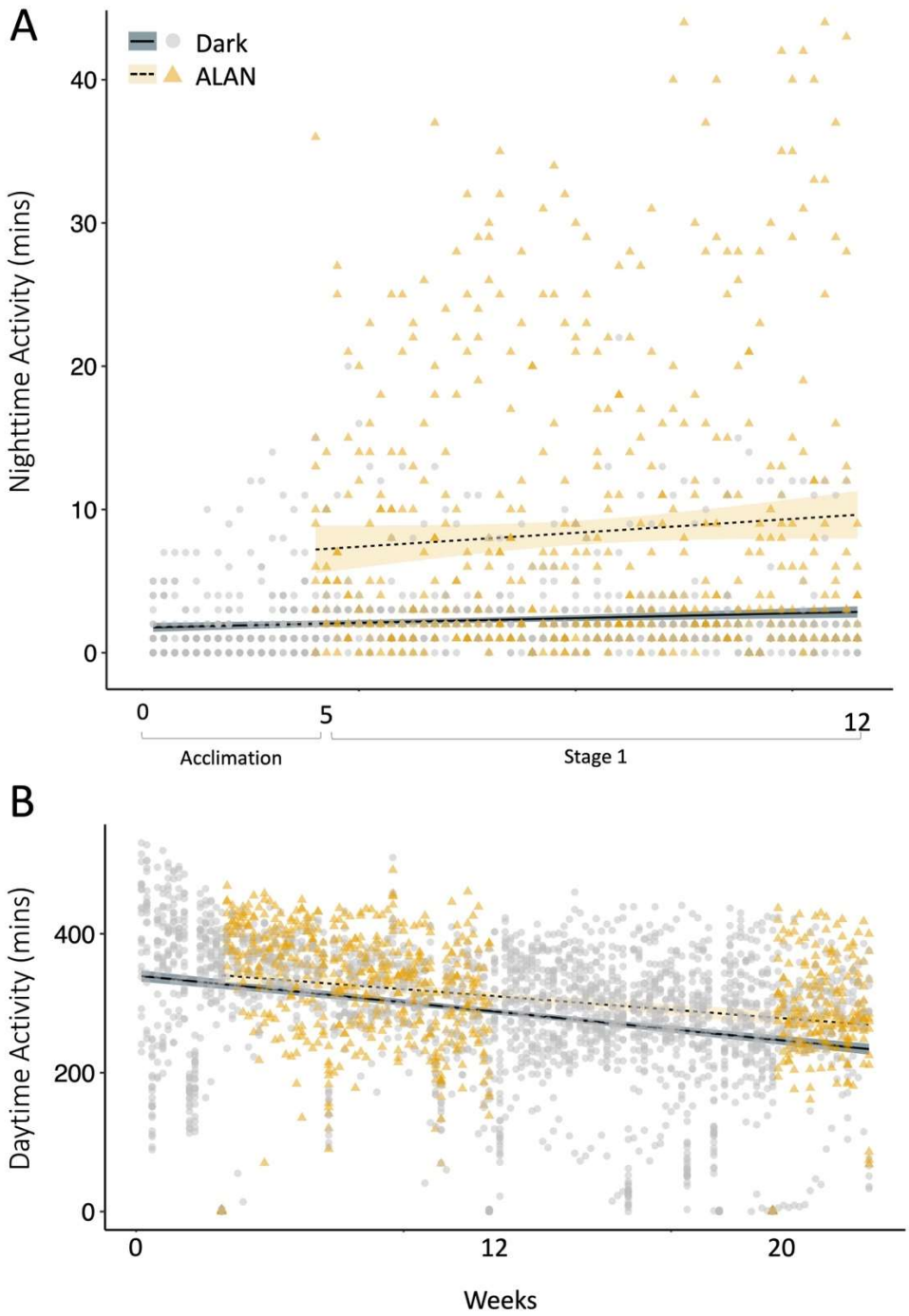

Figure S3. Raw activity data over time. **(A).** In stage 1 (8 weeks), we found that for birds under ALAN, nighttime activity increases slightly over time (day), while birds under dark nights showed consistently low nighttime activity. Points represent raw counts of minutes active per night for all birds (n=11 per ALAN and Dark groups). (**B).** Daytime activity decreased across the duration of experiment, regardless of ALAN exposure.

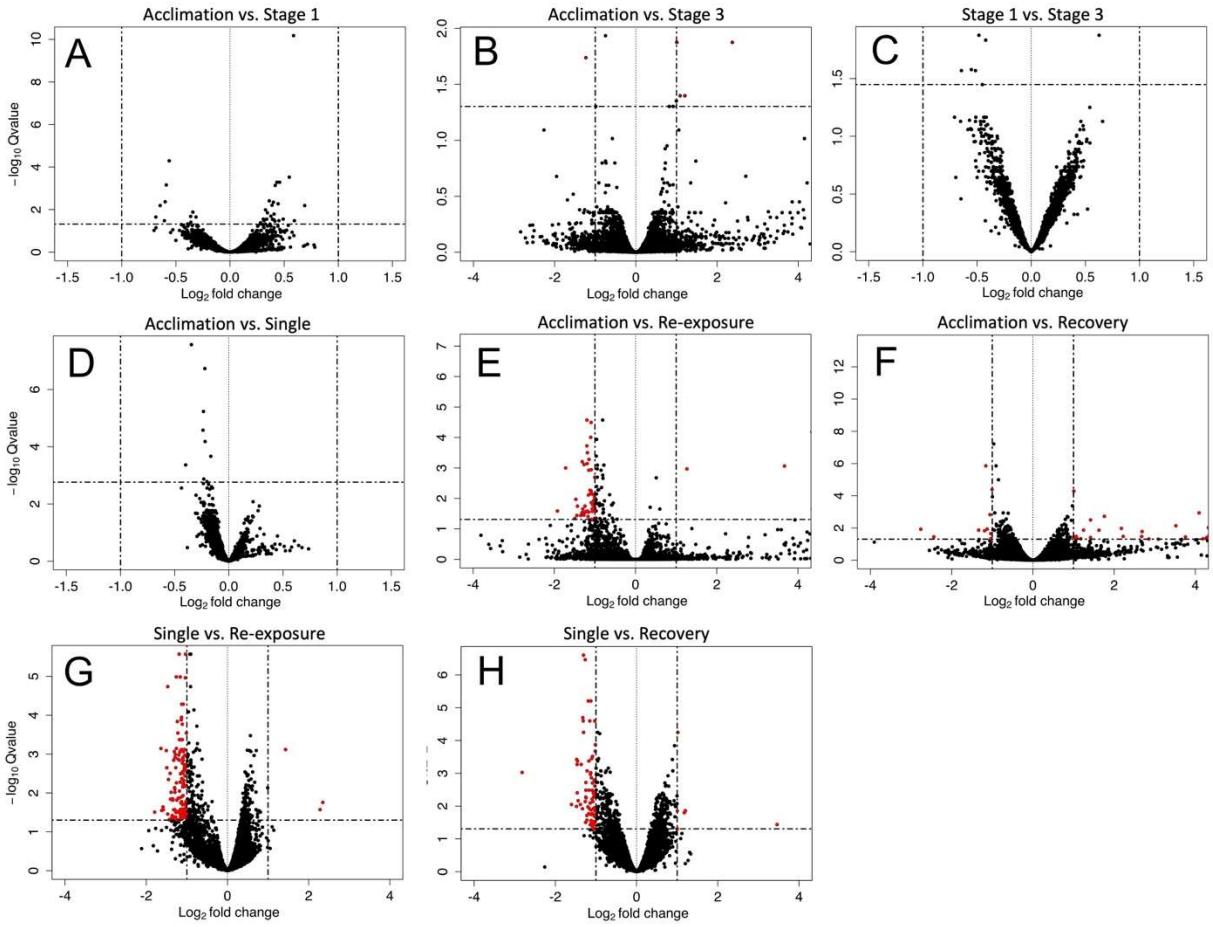

Figure S4. Volcano plots representing differentially expressed genes for comparisons between treatment groups. **(A-C)** Pairwise comparisons between timepoints of our control group, maintained in dark-night conditions for the duration of the experiment, performed to validate the impact of ALAN treatment. **(D-H)** Pairwise comparisons of various groups exposed to ALAN (see Figure 1). "Acclimation" represents birds under dark-night conditions, with no previous ALAN exposure. "Single" represents naïve birds during first ALAN exposures. "Recovery" represents birds returned to dark-night conditions, 8 weeks *after* first ALAN exposures. "Re-exposure" represents birds during secondary exposures to ALAN.

Supplementary References

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