

Elevated DNA Damage without signs of aging in the short-sleeping Mexican Cavefish

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Evan Lloyd, Fanning Xia, Kinsley Moore, Carolina Zertuche, Aakriti Rastogi, Rob Kozol, Olga Kenzior, Wesley Warren, Lior Appelbaum, Rachel L Moran, Chongbei Zhao, Erik Duboue, Nicolas Rohner , Alex C Keene 

Department of Biology, Texas A&M University, College Station, United States • Stowers Institute for Medical Research, Kansas City, United States • Harriet Wilkes Honors College, Florida Atlantic University, Jupiter, United States • Department of Genomics, University of Missouri, Columbia, United States • Faculty of Life Science and the Multidisciplinary Brain Research Center, Bar Ilan University, Ramat Gan, Israel

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Lloyd et al. used an evolutionary comparative approach to study DNA damage repair associated with low sleep duration in *Astyanax mexicanus*, highlighting how the cavefish population has evolved a reduced DNA damage response. The results presented here have **important** implications. Their results are generally **solid** however, the evidence suggesting that sleep differences are linked to DNA damage response is missing and this hypothesis remains to be fully tested.

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Abstract

Dysregulation of sleep has widespread health consequences and represents an enormous health burden. Short-sleeping individuals are predisposed to the effects of neurodegeneration, suggesting a critical role for sleep in the maintenance of neuronal health. While the effects of sleep on cellular function are not completely understood, growing evidence has identified an association between sleep loss and DNA damage, raising the possibility that sleep facilitates efficient DNA repair. The Mexican tetra fish, *Astyanax mexicanus* provides a model to investigate the evolutionary basis for changes in sleep and the consequences of sleep loss. Multiple cave-adapted populations of these fish have evolved to sleep for substantially less time compared to surface populations of the same species without identifiable impacts on healthspan or longevity. To investigate whether the evolved sleep loss is associated with DNA damage and cellular stress, we compared the DNA Damage Response (DDR) and oxidative stress levels between *A. mexicanus* populations. We measured markers of chronic sleep loss and discovered elevated levels of the DNA damage marker γ H2AX in the brain, and increased oxidative stress in the gut of cavefish, consistent with chronic sleep deprivation. Notably, we found that acute UV-induced DNA damage elicited an increase in sleep in surface fish but not in cavefish. On a transcriptional level, only the surface fish activated the photoreactivation repair pathway following UV damage. These findings suggest

a reduction of the DDR in cavefish compared to surface fish that coincides with elevated DNA damage in cavefish. To examine DDR pathways at a cellular level, we created an embryonic fibroblast cell line from the two populations of *A. mexicanus*. We observed that both the DDR and DNA repair were diminished in the cavefish cells, corroborating the *in vivo* findings and suggesting that the acute response to DNA damage is lost in cavefish. To investigate the long-term impact of these changes, we compared the transcriptome in the brain and gut of aged surface fish and cavefish. Strikingly, many genes that are differentially expressed between young and old surface fish do not transcriptionally vary by age in cavefish. Taken together, these findings suggest that cavefish have developed resilience to sleep loss, despite possessing cellular hallmarks of chronic sleep deprivation.

Introduction

Sleep is ubiquitous throughout the animal kingdom and has been identified in animals with relatively simple neural networks, including jellyfish and nematodes, through primates, suggesting ancient function and shared evolutionary origins (Aulsebrook et al., 2016; Joiner, 2016; Keene and Duboue, 2018; McNamara et al., 2009; Zimmerman et al., 2008). While the primary functions of sleep are not fully understood, it is essential for many processes including neural connectivity, clearance of toxic metabolites, immunity, learning, and memory (Frank, 2020; Hartmann, 1973; Siegel, 2005). There is growing evidence that DNA damage may play an important role in sleep drive (Carroll et al., 2016; Vaccaro et al., 2020; Zada et al., 2021, 2019). DNA damage is associated with periods of prolonged wakefulness and is reduced during sleep across numerous species, including *C. elegans*, zebrafish, mice, and humans (Bellesi et al., 2016; Cheung et al., 2019; Goetting et al., 2020). In turn, sleep disruption is associated with DNA damage, and sleep deprivation (SD) inhibits the expression of DNA repair genes in humans (Carroll et al., 2016; Goetting et al., 2020) suggesting a critical role for sleep in the maintenance of genome integrity and function and an association between sleep loss and DNA damage, which could lead to neurodegeneration. Further, sleep loss results in elevated reactive oxygen species (ROS), a known mediator of DNA damage, in the gut and/or brain that contribute to mortality in *Drosophila* (Haynes et al., 2024; Vaccaro et al., 2020). The effects of sleep loss on ROS production in the gut is also present in mice (Vaccaro et al., 2020). Despite these advances, little is known about the cellular consequences of sleep loss or the evolutionary relationship between DNA damage and sleep regulation.

Comparative approaches examining evolutionarily derived differences in sleep have provided significant insight into the genetic and functional basis of sleep regulation (Allada and Siegel, 2008; Cirelli, 2009; Zimmerman et al., 2008). While the majority of sleep studies in fish have used zebrafish, the Mexican tetra *Astyanax mexicanus* is an emerging model for investigating the genetic and evolutionary basis underlying behavioral and physiological traits (Keene et al., 2015; Keene and Appelbaum, 2019; Kowalko, 2020; McGaugh et al., 2020a; Yoshizawa, 2015). *A. mexicanus* exists as blind cave populations and an extant surface population; while the surface and cave populations are geographically isolated, they remain interfertile and capable of hybridization in natural and laboratory settings (Moran et al., 2023). In this system, there are similar sleep loss phenotypes among geographically and geologically isolated cave populations (Aspiras et al., 2015; Duboué et al., 2011) with likely unique genetic bases between caves (Duboué et al., 2011; Mack et al., 2021; Yoshizawa et al., 2015). Further, evolved differences in DNA repair genes have been identified across all three cave populations studied to date, including links between mechanisms regulating sleep, light responsiveness, and DNA repair (Beale et al., 2013; Mack et al., 2021). These findings support the notion that the genetic and molecular underpinnings of sleep are closely related to DNA repair processes in cavefish.

Examining the ecological factors that contribute to evolved changes in sleep regulation and the physiological consequences of this sleep loss has the potential to address the fundamental functions of sleep. For example, in humans, insomnia is associated with many different diseases and increased morbidity, suggesting that sleep is critical for healthy aging (Arble et al., 2015; Carroll and Prather, 2021). Strikingly, cavefish sleep as little as 1-2 hours per day, in contrast to their surface counterparts, which sleep as much as 6-10 hours a day (Duboue et al., 2011). Despite the dramatic reduction in sleep, there are no apparently health consequences to cavefish, suggesting an established resilience to sleep loss (Cobham and Rohner, 2024; Riddle et al., 2018a; Rohner, 2018). Considering that changes in the levels of DNA breaks are associated with sleep regulation in flies, zebrafish, mice, and humans (Bellesi et al., 2016; Carroll et al., 2016; Zada et al., 2021), it is possible that intrinsic changes in the DNA repair and DDR pathways underlie reduced sleep need in cavefish.

Here, we sought to investigate the relationship between DNA damage and the evolution of sleep, to test at the cellular and organismal levels whether cavefish have markers of chronic sleep deprivation and accelerated aging. We find that DNA damage is elevated in cavefish brains, consistent with the notion that sleep loss is associated with elevated levels of DNA damage. The transcriptional and behavioral response to UV damage is blunted in cavefish, and cavefish cells exhibit diminished DNA repair capabilities raising the possibility that reduced DDR function may contribute to sleep loss in cavefish. To examine the long-term consequences of reduced DDR, we examined transcriptional differences in young and aged surface and cavefish. While aging in surface fish is associated with broad transcriptional changes across tissue types, there are relatively few transcriptional differences between young and aged cavefish. Together, these findings reveal that cavefish appear to have developed molecular resilience to aging despite elevated DNA damage that likely derives from sleep loss.

Results

In vertebrates, including teleost fish such as zebrafish and *A. mexicanus*, DNA damage activates a highly conserved and stereotypical response and repair program. Markers of DDR proteins can be localized and quantified at the cellular and whole organism levels. Phosphorylation of histone H2AX on serine 139 (γ H2AX) offers a well-established and quantifiable biomarker of cellular response to DNA double-strand breaks that is necessary for the assembly of repair complexes (Siddiqui et al., 2015). We compared γ H2AX between surface fish and Pachón cavefish brains at ZT0, a time when both populations are behaviorally active and sleep is minimal (Fig 1A–B and Supplemental Movie 1). Levels of γ H2AX were elevated in the rhombencephalon, telencephalon, and mesencephalon of cavefish compared to surface fish (Fig 1C). These findings suggest that DNA damage is elevated in short-sleeping cavefish compared to their surface fish counterparts.

Sleep loss is associated with reduced gut function, including the accumulation of reactive oxygen species (ROS) (Vaccaro et al., 2020). To examine whether markers of sleep loss extend to the gut in *A. mexicanus*, we compared ROS in the guts of surface and cavefish. Fish aged 6 days post fertilization (dpf) were incubated in the ROS marker dihydroethidium (DHE), and guts were imaged on a confocal microscope. We found that ROS in the gut is upregulated in cavefish, reinforcing the idea that cellular stress and canonical markers of DDR are elevated in these short-sleeping fish (Fig 1D–G); ROS levels were elevated in the stomach, midgut, and hindgut, but not in the rectum (Fig 1G). Prior to imaging, both surface and cavefish had been reared in a temperature-controlled incubator, and relied solely on their yolk sac for nutrients; so, differences in gut ROS cannot be attributed to differences in rearing or feeding conditions. Together, these findings fortify the notion that cellular stress is elevated in the gut of cavefish relative to surface fish.

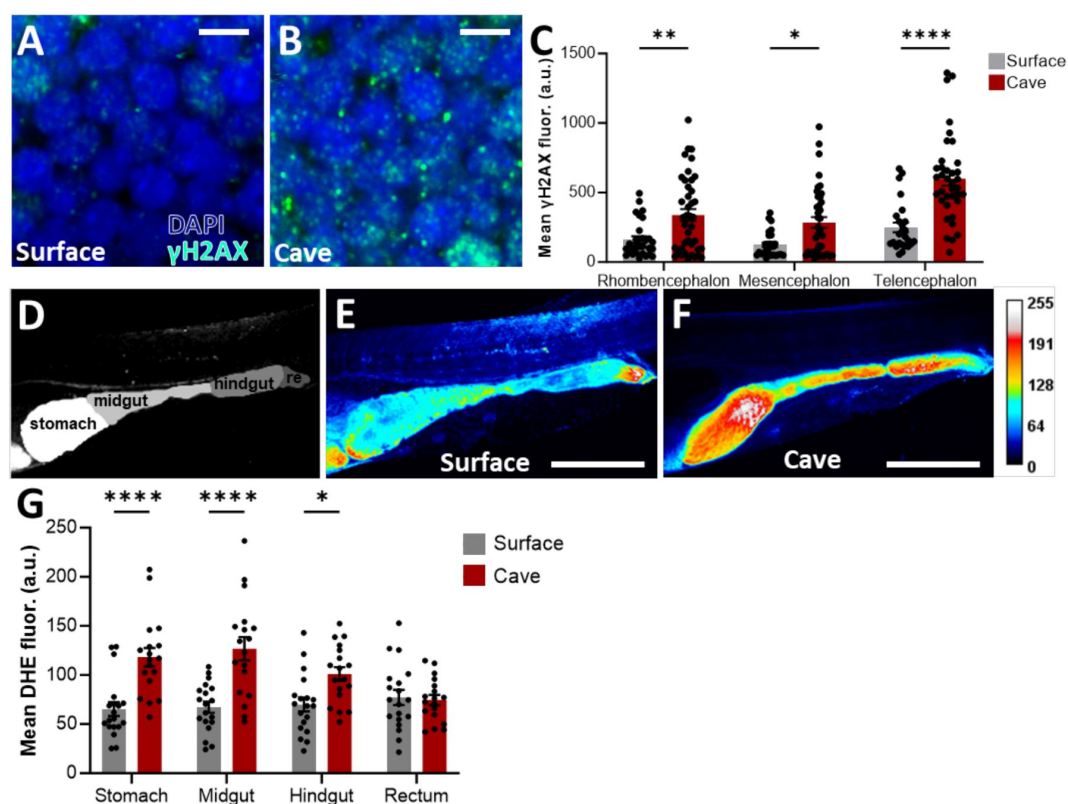


Figure 1.

Cavefish harbor increased neuronal DNA damage and gut ROS.

(A, B) Representative images of cells stained with DAPI and γH2AX in the rhombencephalon of surface (A) and cavefish (B). Scale bar = 5 μm. (C) Mean γH2AX fluorescence across three regions of surface and cavefish brains. (Mixed-effects analysis: $F_{1,68} = 32.08$, $p < 0.0001$). (D) Representative image of larval gut showing regions in false color (re: rectum). (E, F) representative images of surface and cave guts stained with DHE marking ROS. Scale bar = 500 μm. (G) Mean DHE fluorescence across four regions of surface and cavefish guts (two-way repeated measures ANOVA: $F_{1,35} = 48.36$, $p < 0.0001$).

To further examine the direct link between DNA damage and sleep in *A. mexicanus*, surface fish and cavefish were exposed to short periods of UV-B radiation, known to cause DNA damage and inducing double-stranded breaks (Zada et al., 2021 [↗](#)). In surface fish, UV exposure resulted in a dose-dependent increase in sleep for up to three hours, similar to findings in zebrafish [10] (**Fig 2A** [↗](#) and **2B** [↗](#)). Interestingly, sleep decreased in surface fish sleep during the nighttime, perhaps the result of sleep credit that derives from increased sleep during the day (**Fig 2A** [↗](#)) (Öztürk-Çolak et al., 2020 [↗](#)). Increases in sleep amount in surface fish were mediated by both increased bout number, and, at the higher dose, an increase in bout length (**Fig 2C** [↗](#) and **2D** [↗](#)). Conversely, there was no effect of UV treatment on daytime or nighttime sleep in cavefish (**Fig 2E–H** [↗](#)).

Analysis of sleep- and wake-probability was consistent with these measurements, showing elevated sleep pressure and reduced wake pressure in surface fish during the three hours following treatment; in cavefish, there was no change (**Fig S1** [↗](#)). These results confirm that UV-induced DNA damage promotes sleep in *A. mexicanus* surface fish, whereas this response is lost in cavefish.

We examined the effects of the high dose (60 seconds) of UV treatment at zeitgeber time (ZT) 0 (onset of lights on) on the transcriptional response in surface fish and cavefish. Fish aged 6 dpf were harvested for RNA extraction 90 minutes following UV exposure (**Fig 3A** [↗](#), Supplemental Data 1). PCA analysis of RNA expression showed that the largest factor driving variability in transcriptional response across samples was population, with principal component 1 separating samples by population and accounting for 86% of the variance. Principal component 2 separated samples by treatment and accounted for 9% of the variance (**Fig 3B** [↗](#)). Analysis revealed numerous genes that were differentially expressed in both populations, including upregulation of the RNA Polymerase regulating transcription factor *fosl1a* and downregulation of *spi-c* (**Fig 3C** [↗](#)). Numerous genes were selectively differentially expressed, including upregulation of the heat shock protein *hspb9* in surface fish and downregulation of the glucose sensor *gck* in cavefish (**Fig 3C** [↗](#) and **Fig S2A,B** [↗](#)). To determine if the DDR pathway is activated in cavefish following exposure to UV light, we quantified changes in pathway components in surface fish and cavefish. A heat map of DNA repair genes significantly upregulated in surface fish revealed that several components of DNA repair pathways are differentially expressed in cavefish following UV treatment (**Fig 3D** [↗](#)). Notably, cyclobutane pyrimidine dimer photolyase (*cpdp*), an important component of the photoreactivation repair pathway for UV-induced DNA damage, responds strongly in surface fish, but fails to respond in cavefish. Although previous groups have reported that *cpdp* is constitutively upregulated in adult cavefish (Beale et al., 2013 [↗](#)), our analysis did not align with this finding; however, this could be due to differences in the age or circadian timepoint studied. A more detailed analysis of previous circadian transcriptomic studies in cavefish revealed that two of the DDR genes are elevated at some, but not all phases of the circadian cycle (**Fig S3** [↗](#)). Additionally, *xrcc3*, a paralog of human *rad51* that is important for homologous recombination, and *fan1*, a component of the Fanconi Anemia pathway, are nonresponsive to UV-B treatment in cavefish. Conversely, *ube2al*, which is required for post-replication DNA repair, is perhaps even more strongly activated in cavefish compared to surface fish. Together, these findings suggest that DNA repair processes in cavefish have undergone complex changes, with some pathways rendered nonfunctional, while others may have been upregulated to compensate for loss of function in other areas. To understand transcriptional changes more fully as a result of UV-B treatment, gene set enrichment analysis (GSEA) was performed on both surface fish and cavefish. A large number of pathways were enriched in both populations (**Fig S2C** [↗](#) and **S2D** [↗](#)); to examine differences between the transcriptional responses of the two populations, we identified pathways which were specifically enriched in either surface fish or cavefish (**Fig 3E** [↗](#)). Only surface fish showed significant activation of genes associated with response to ROS and cell redox homeostasis, consistent with measurements of elevated ROS in cavefish, whereas cavefish showed activation of genes associated with metabolic processes and suppression of genes associated with synaptic signaling, suggesting altered responses to DNA damage.

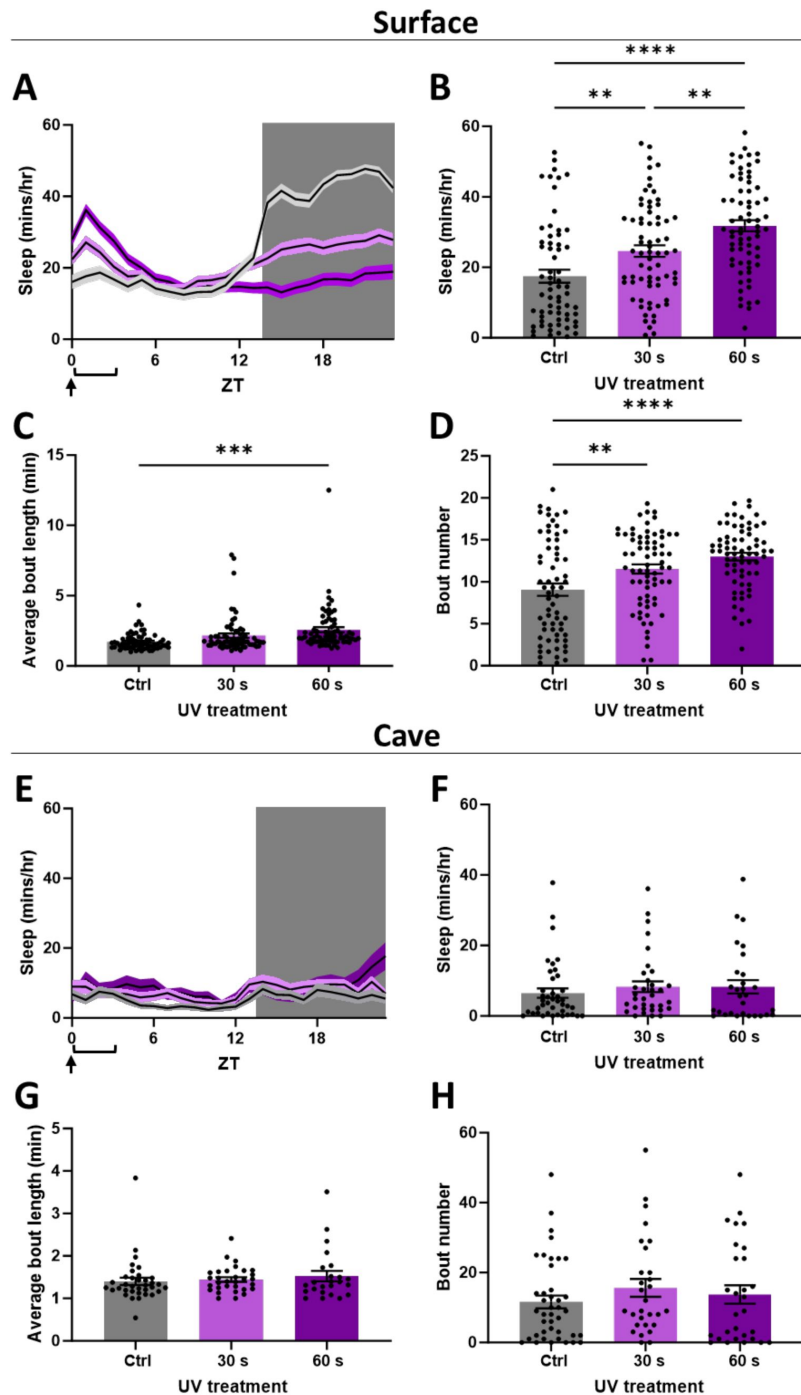


Figure 2.

Cavefish lack a sleep response to UV-B induced DNA damage.

(A) 24-hour sleep profiles of surface fish exposed to 30 or 60 seconds of UV-B light, compared to controls. (B) Average sleep amount in surface fish in the 3 hours following UV-B exposure (one-way ANOVA: $F_{2, 202} = 18.75$, $p < 0.0001$). (C) Average bout length in surface fish in the 3 hours following UV-B exposure (one-way ANOVA: $F_{2, 201} = 8.301$, $p = 0.0003$). (D) Bout number in the 3 hours following UV-B exposure (one-way ANOVA: $F_{2, 201} = 11.5$, $p < 0.0001$). (E) 24-hour sleep profiles of cavefish exposed to 30 or 60 seconds of UV-B light, compared to controls. (F) Average sleep amount in cavefish in the 3 hours following UV-B exposure. (G) Average bout length in cavefish in the 3 hours following UV-B exposure. (H) Bout number in the 3 hours following UV-B exposure. (Arrows indicate time of UV treatment; brackets indicate time period used for statistical comparisons of sleep.)

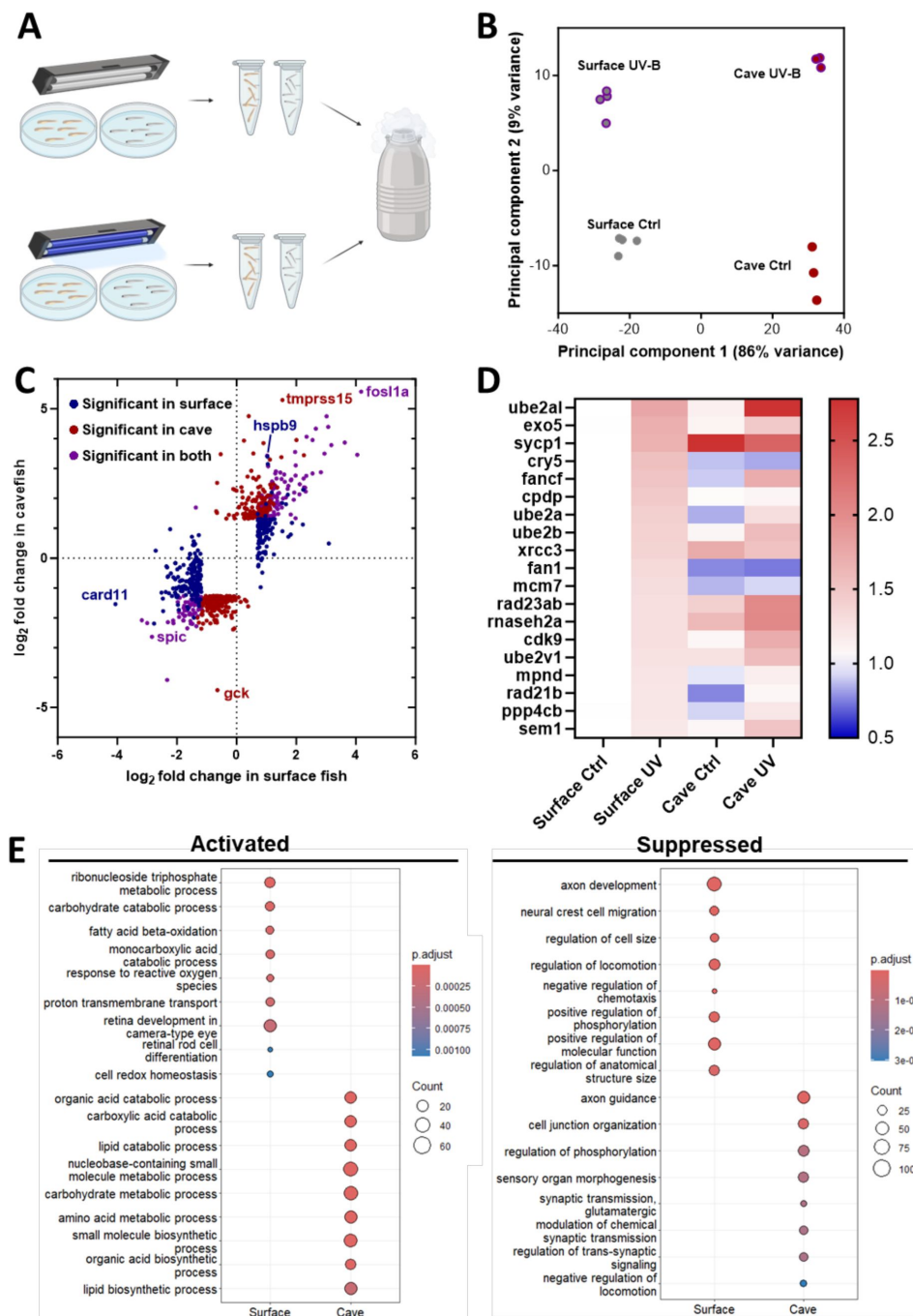


Figure 3.

Transcriptional responses to UV-B-induced DNA damage in surface fish and cavefish.

(A) Schematic of experimental design. Surface and cave larvae were subjected to 60 seconds of UV-B radiation, left undisturbed for 90 minutes, and then flash-frozen in liquid nitrogen in groups of 6 for RNA extraction. **(B)** Multidimensional scaling plot depicting the variances in principal component space between the processed sequencing samples. PC1 (two-way ANOVA: (Treatment) $F_{1,10}=6.388$, $p=0.03$, (Population) $F_{1,10}=4970$, $p<0.0001$, (Interaction) $F_{1,10}=17.56$, $p=0.0019$). PC2 (two-way ANOVA: (Treatment) $F_{1,10}=465.0$, $p<0.0001$, (Population) $F_{1,10}=0.4969$, $p=0.497$, (Interaction) $F_{1,10}=18.56$, $p=0.0015$). **(C)** Bi-directional volcano plot of changes in gene expression in surface and cave larvae after exposure to DNA damaging UV-B radiation. **(D)** Heat map of gene expression in DNA repair genes which responded significantly in UV-B-exposed surface fish. Each cell is normalized to its respective surface control group. **(E)** Dot plot visualizing GO terms which were significantly enriched in only surface fish or cavefish after exposure to UV-B radiation.

To quantify DNA damage on a cellular level, we established embryonic fibroblast cell lines derived from surface fish and Pachón cavefish embryos (**Fig S4A** [↗](#)). In brief, we dissociated and sterilized cells from embryos less than 12 hours post fertilization and isolated individual clones that were propagated for over 40 passages. To confirm that the cell lines indeed represented fibroblasts, we stained the cells for the presence of vimentin, a known fibroblast marker ([Satelli and Li, 2011](#) [↗](#)). Both cell lines exhibited stable vimentin expression (**Fig S4B** [↗](#)). We further validated the fibroblast nature of the cell lines by RNA sequencing, which showed enhanced expression of other fibroblast signature genes such as *col1a1* compared to other cell types, including the previously established liver-derived cell lines ([Krishnan et al., 2022](#) [↗](#)) from the same species, mouse stem cells, and mouse embryonic fibroblasts ([Wang et al., 2019](#) [↗](#)) (**Fig S4C** [↗](#)).

We next used the newly generated fibroblast cell lines to measure DNA damage level upon UV radiation exposure. We exposed the cells to 100 J/m² UV radiation and visualized UV-induced DNA lesions using an antibody targeting cyclobutene pyrimidine dimer (CPD) as a DNA damage marker. Both the surface fish and cavefish-derived cell lines exhibited strong CPD induction, indicating pronounced UV-induced DNA damage (**Fig 4A** [↗](#)). We quantified the mean fluorescence per nucleus area using the Cellpose function ([Stringer et al., 2021](#) [↗](#)) of ImageJ and found no significant difference between the two derived cell lines ($p = 0.6404$, two-way ANOVA, **Fig 4B** [↗](#)).

To assess whether a similar level of DNA damage in the different cell lines results in variations in DNA damage repair, we quantified γ H2AX levels after UV treatment. Measurements were taken bi-hourly for 6 hours post exposure. The surface fish cells demonstrated a marked increase in γ H2AX, whereas the cavefish cell lines showed only a modest rise, implying a diminished UV damage repair capability in cavefish cells (**Fig 4C** [↗](#) and **4D** [↗](#)). To validate these results, we repeated these experiments on liver-derived cell lines ([Krishnan et al., 2022](#) [↗](#)) and observed a similar trend of reduced levels of γ H2AX after radiation exposure in cavefish-derived cells, while the surface fish cells showed strong induction (**Fig S4D** [↗](#)). This pattern suggests that the DDR differences are not confined to tissue type. To directly test the ability to repair DNA, we employed a host cell reactivation assay ([Nagel et al., 2014](#) [↗](#); [Tian et al., 2019](#) [↗](#)). Briefly, green fluorescent protein (GFP) plasmid was treated with 600 J/m² UV. We transfected the cell lines with either an intact or the *in vitro* UV damaged GFP plasmid and tracked GFP fluorescence recovery after 50 hours using flow cytometry as a proxy for the ability of the host cell to repair the plasmid (**Fig 4G** [↗](#)). Consistent with the γ H2AX findings, the UV-damaged plasmid transfected cavefish cells displayed a substantially lower GFP signal recovery (~22% relative to control) than the surface fish cells (~49% relative to control) (**Fig 4E** [↗](#) and **4F** [↗](#)). These observations, indicating a diminished DNA repair capacity, align with the sleep behavior differences we observed in UV-treated fish larvae.

DNA damage in the brain, ROS in the gut, and sleep loss are associated with aging ([Carroll and Prather, 2021](#) [↗](#); [Maynard et al., 2015](#) [↗](#); [Schumacher et al., 2021](#) [↗](#)). While *A. mexicanus* cavefish have evolved many traits that would be detrimental to humans or other species there is evidence that they have also evolved metabolic and physiological resilience, enabling them to enjoy a similar or even extended lifespan compared to surface fish ([Lunghi and Bilandžija, 2022](#) [↗](#); [Medley et al., 2022](#) [↗](#); [Riddle et al., 2018a](#) [↗](#)). To examine the effects of long-term accumulation of DNA damage in the brain, elevated gut ROS, and sleep loss, we examined the transcriptional profiles of tissue in young and aged fish. Briefly, we dissected the brain, gut, liver, muscle, and heart of young (~1 year old) and aged (7–8 year old) surface fish and Pachón cavefish. PCA analysis of brain samples revealed a strong contribution of population to the sample variance, with samples separated by population across PC1 and accounting for 51% of the variance (**Fig 5A** [↗](#)). Interestingly, while surface fish samples were separated by age across PC2, there was no separation of samples by age in cavefish (**Fig 5A** [↗](#)). The same trend held true for gene expression in the gut (**Fig S5A** [↗](#)). PCA plots of gene expression in the heart and liver did not show clear separation across either population or age, while muscle tissue showed separation by population, but not age (**Fig S5B** [↗](#)–**D** [↗](#)). To examine the impacts on the broader transcriptome, we compared

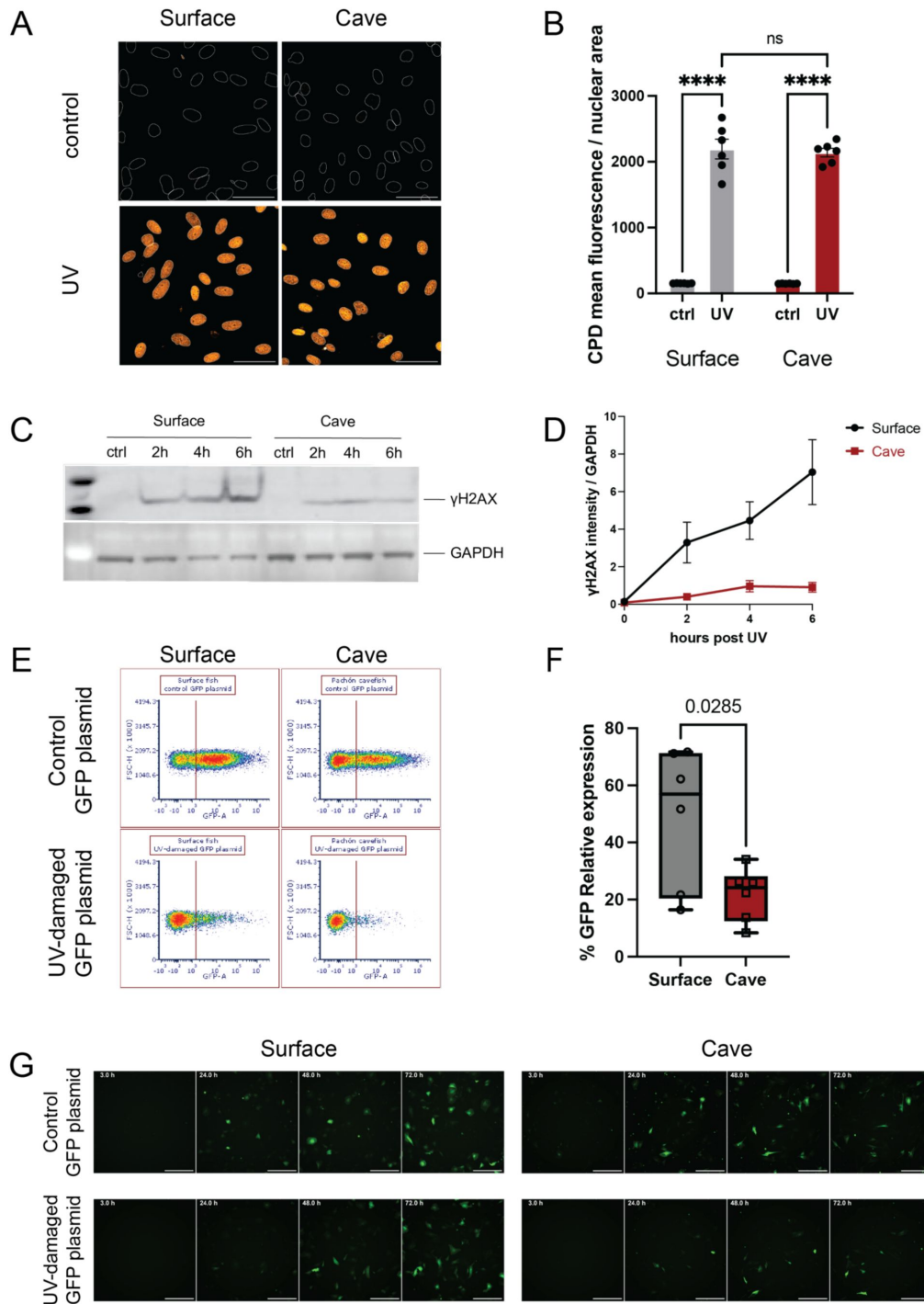


Figure 4.

Pachón cavefish-derived cells exhibit a lower UV-induced DNA damage response and repair compared to surface fish. **(A,B)** Immunostaining of cyclobutene pyrimidine dimer (CPD) shows a similar DNA damage level induced by UV in surface fish (grey) and Pachón cavefish (red) embryonic fibroblasts. White circles indicate the nuclear area by DAPI staining. Orange indicates CPD. Scale bar, 40 μ m. *P*-values were determined by two-way ANOVA: $F = 0.09703$, $p = 0.7586$. ns $p = 0.6404$, **** $p < 0.0001$. **(C,D)** Western blot of γ H2AX indicates a diminished DNA damage response in Pachón cavefish embryonic fibroblast compared to surface fish cells. **(E,F)** Flow cytometry images and quantification for host cell reactivation assays in surface fish and Pachón cavefish embryonic fibroblast. Red line sets the GFP positive signal threshold. *P*-values were determined by unpaired *t*-test. **(G)** Representative GFP images for host cell reactivation assays in surface fish and Pachón cavefish embryonic fibroblast. Scale bars, 500 μ m.

the number of differentially expressed genes between young and aged populations of surface fish and cavefish. Across all tissues, there were markedly more transcripts that were differentially expressed between young and aged surface fish than cavefish in the brain, gut, heart, liver, and muscle (**Fig 5B** [↗](#)). Together, these findings reveal that the transcriptome of cavefish is resilient to age-associated changes despite sleep loss, elevated ROS, and elevated DNA damage.

We sought to examine the specific genes that were differentially expressed between surface fish and cavefish, providing potential mechanisms of resilience to DNA damage and sleep loss. Because sleep is considered necessary, specifically for repair of neuronal DNA damage, we first examined transcriptional differences in the aging surface fish and cavefish brains. Within the brain, there were only five genes which showed significant changes in both surface fish and cavefish; among these was the gene *top2a*, which is reduced in both populations (**Fig 5C** [↗](#)). *Top2a* is considered essential for structural maintenance of chromosomes during cell division. Intriguingly, GSEA analysis did not reveal any significantly enriched pathways in aged surface fish brains despite the high number of differentially regulated genes, while the aged cavefish brains showed suppression of gene sets related to chromosome condensation and segregation (**Fig 5D** [↗](#)). These processes are known to deteriorate with age because of unrepaired DNA damage, particularly in the brain (Barroso-Vilares et al., 2020 [↗](#)). Across non-brain tissues, we found enrichment for a wide variety of processes, some which overlapped across tissues and populations and some which did not, consistent with the idea that aging is a complex process governed by many factors (**Fig S6** [↗](#)). Taken together, these results indicate that, despite elevated levels of DNA damage and impaired DNA damage and repair mechanisms, cavefish are at least partially protected from their harmful effects, and exhibit reduced transcriptional changes during aging.

Discussion

We have investigated differences in DNA damage and the DDR pathway in *A. mexicanus*, a model for evolved sleep loss. UV and other agents that induce DNA damage promote sleep in diverse animals, suggesting a fundamental and highly conserved relationship between DNA damage and sleep regulation (Bellesi et al., 2016 [↗](#); DeBardeleben et al., 2017 [↗](#); Zada et al., 2021 [↗](#)). Similarly, we find that DNA damage in the brain and ROS levels in the gut are elevated in Pachón cavefish compared to surface fish. These findings are consistent with the phenotypes of sleep-deprived invertebrates and mammals, supporting the notion that cavefish are sleep deprived (Bellesi et al., 2016 [↗](#); Vaccaro et al., 2020 [↗](#)). Beyond the Pachón cavefish population, the two other larval cavefish populations tested to date have reduced sleep compared to surface fish (Duboué et al., 2011 [↗](#); Jaggard et al., 2020 [↗](#)). Further investigation of DNA damage in these populations is necessary to determine whether the cellular effects of sleep loss are conserved in independently evolved cavefish populations (Moran et al., 2022 [↗](#); Yoshizawa et al., 2015 [↗](#)). There are many species that have evolved sleep loss, particularly in defined ecological contexts, including newborn cetaceans that forgo sleep, the Arctic tern that suppresses sleep during the mating season, and frigate birds that have reduced and unilateral sleep during prolonged flight (Lesku et al., 2012 [↗](#); Lyamin et al., 2005 [↗](#); Rattenborg et al., 2016 [↗](#)). It will be highly informative to investigate the presence of DNA damage and other markers of sleep loss within a natural ecological context and in evolutionary models with altered sleep.

Multiple studies have now revealed changes in sleep, locomotor behavior and DNA damage across different species of cavefish (Keene et al., 2024 [↗](#)). The adaptive value of these changes in a natural setting, and their impact on longevity remain poorly understood. Additional experiments are necessary to address the cellular relationship between sleep and DNA damage. In zebrafish, sleep loss induces DNA damage, while inducing DNA damage promotes sleep (Zada et al., 2021 [↗](#)). These findings suggest DNA damage contributes to sleep homeostasis. We have previously shown that cavefish rebound following sleep deprivation, suggesting they maintain an intact sleep homeostat (McGaugh et al., 2020b [↗](#)).

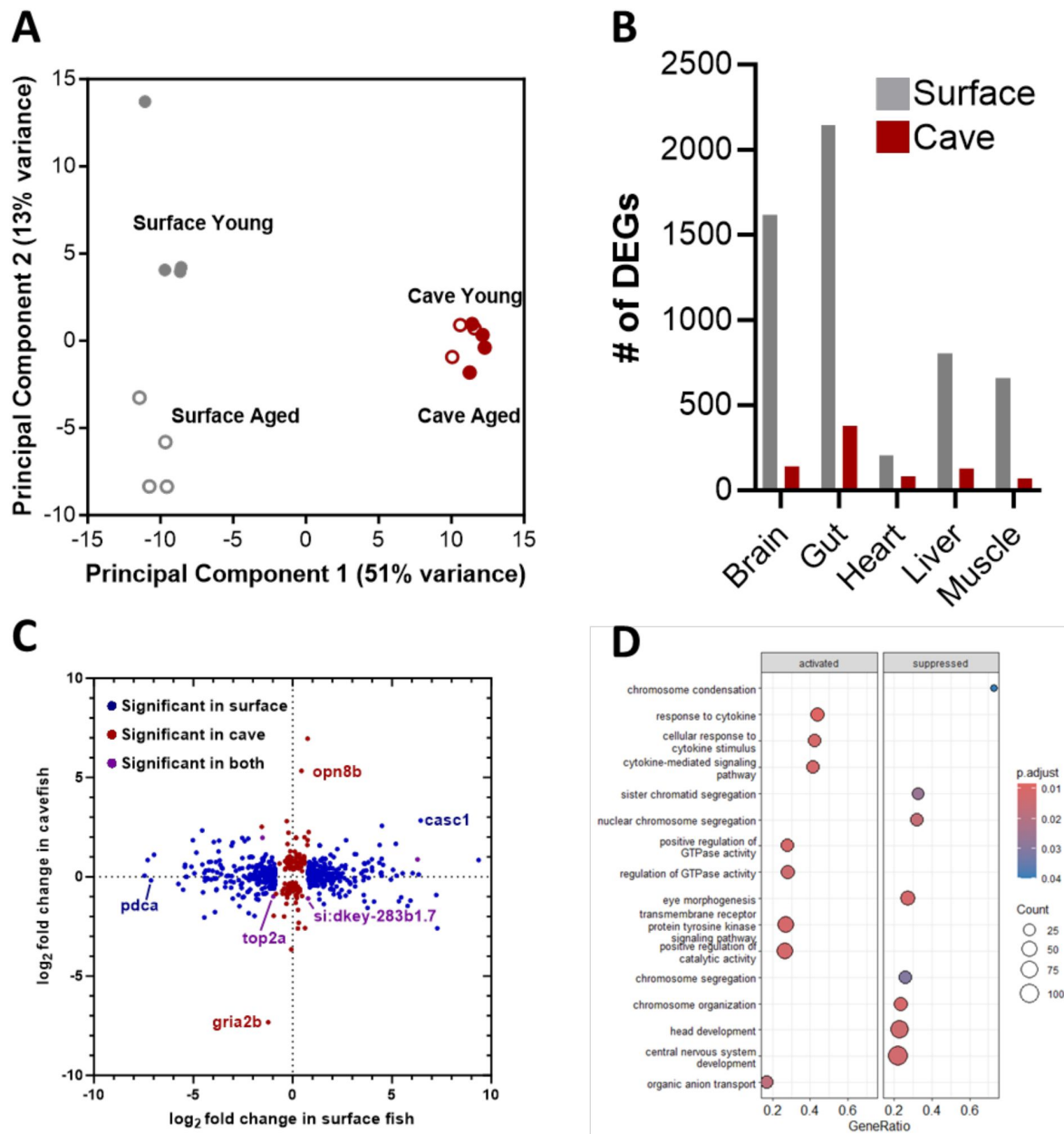


Figure 5.

Transcriptional response to aging is diminished in cavefish across tissues.

(A) Multidimensional scaling plot plotting the distances in principal component space between the brain samples. PC1 (two-way ANOVA: (Treatment) $F_{1,11}=4.209$, $p=0.0648$, (Population) $F_{1,11}=2133$, $p<0.0001$, (Interaction) $F_{1,11}=0.029$, $p=0.867$ PC2 (two-way ANOVA: (Treatment) $F_{1,11}=16.83$, $p=0.0018$, (Population) $F_{1,11}=0.0002$, $p<0.99$, (Interaction) $F_{1,11}=19.37$, $p=0.0011$ (B) Number of differentially expressed genes in the aged condition across tissues. (C) Bi-directional volcano plot depicting differences in gene expression between young and aged brains. (D) Dot plot visualizing the top 8 activated and suppressed gene ontology terms in aged cavefish brains resulting from GSE analysis.

Our cellular analysis further revealed a muted DDR in cavefish, which likely contributes to the increased DNA damage noted *in vivo*. Since photolyases are primarily utilized for the repair of UV-induced cyclobutene pyrimidine dimers, and their repair processes are also dependent on light input (Liu et al., 2011 [↗](#)), it is plausible that these genes were not favored by natural selection in cavefish, leading to accumulated mutations and a loss of functional DNA damage response. This hypothesis is supported by findings that Somalian cavefish have lost critical DNA repair enhancers needed for an induced DDR (Zhao et al., 2018 [↗](#)). The fact that Mexican cavefish retain some capacity for light-induced DNA repair might be attributed to their relatively recent divergence from their surface-dwelling counterparts, estimated to be less than 200,000 years ago (Fumey et al., 2018 [↗](#); Herman et al., 2018 [↗](#)). Although our findings are consistent with studies on DNA repair in other cavefish species, they contrast with previous research suggesting an increased DNA repair function in certain *Astyanax* cavefish populations (Beale et al., 2013 [↗](#)). While increased expression of some DNA repair genes is also observed in our study (Fig S3 [↗](#)), our cellular assays demonstrate that this does not equate to enhanced DNA repair activity. However, our analysis focused only on two different cell lines (embryonic fibroblasts and liver-derived cells), while the previous study looked at DNA repair in fins (Beale et al., 2013 [↗](#)). Further research will be required to resolve these differences and fully understand DNA repair dynamics in cavefish.

In *Drosophila* and mice, both acute and chronic sleep loss is associated with reduced longevity, and it is postulated that ROS-associated gut dysregulation leads to death in chronically sleep-deprived individuals (Vaccaro et al., 2020 [↗](#)). Therefore, it is interesting that cavefish do not exhibit clear signs of accelerated aging compared to surface fish (Medley et al., 2022 [↗](#); Riddle et al., 2018a [↗](#)). The lifespan of surface and cave populations of *A. mexicanus* is reported to exceed 20 years, largely preventing the use of longevity as a readout for aging (Rohner, personal communication). However, DNA damage and aging-associated transcriptional changes can provide a proxy for biological aging. The findings that the transcriptional architecture of cavefish does not vary to the degree of surface fish indicates a form of genomic stability or a decelerated aging process, which could be a focal point for future research into the mechanics of aging and its relationship with sleep and DNA repair. In addition to sleep loss, cavefish have fatty livers, reductions in heart regeneration, and chronically elevated blood glucose (Aspiras et al., 2015 [↗](#); Riddle et al., 2018a [↗](#)). These findings raise the possibility that cavefish may have evolved a broad range of resilience mechanisms to biological stress. Consistent with this notion, cavefish have evolved a reduced metabolic rate and elevated metabolites associated with hypoxia and longevity (Medley et al., 2022 [↗](#)). Many of these features, such as reduced metabolic rate, are present in other long-lived organisms, including the naked mole-rat (*Heterocephalus glaber*) and the cave olm (*Proteus anguinus*) (Lunghi and Bilandžija, 2022 [↗](#)). Comparing differences between surface fish and cavefish, or similarities between cavefish and other long-lived models, may provide a system to study resilience to biological stress.

Taken together, these findings suggest that cavefish can be used as a model to study evolved loss of DDR and biological resilience. Growing genetic and genomic tools in cavefish including multiple chromosome-level sequenced genomes and single-cell RNA sequencing atlases may allow for the identification of markers of selection for DNA repair and cell-type specific transcriptional changes in sleep-regulating neurons. In addition, the study of DNA damage and ROS in additional populations of cavefish provides the opportunity to identify multiple mechanisms of DNA damage response.

Material and Methods

Fish husbandry

Fish used in behavioral, immunohistochemistry, and UV-B experiments were generated and raised at Texas A&M University in dedicated aquarium facilities, with water temperature maintained at 23°C on a 14 hour light : 10 hour dark schedule as previously described (Kozol et al., 2023 [DOI](#)). To stimulate breeding, water temperature was raised by ~2°C with a submersible heater, and fish were fed frozen blood worms to satiation 2–3 times daily (Elipot et al., 2014 [DOI](#)). Following breeding, embryos were collected from tanks and raised in a temperature-controlled incubator under the same temperature and light cycle until used for experiments. Fish used for tissue dissections were collected from aquarium facilities at Florida Atlantic University, maintained under the same conditions. Fish and embryos used for cell line derivation were collected from the cavefish facility at the Stowers Institute for Medical Research, maintained under similar conditions, except for the food. Adult fish were fed with Mysis shrimp once in the morning and Gemma 800 (Skretting, Gemma Silk) once in the afternoon. Fish in the breeding cycle were fed with Gemma 800 once at noon.

Fibroblast cell line derivation

Embryos were collected after spawning and were dechorionated before 14 hours post fertilization (hpf). Embryos were then washed in washing media (PBS supplemented with 50 U/ml Penicillin + 0.05 mg/ml Streptomycin) for 30 min three times. Five embryos were transferred to a sterile 1.5 ml tube with 0.5 ml of wash media where the yolk sac of embryos was removed by pipetting up and down ~10 times with a 200 µl pipette tip. Tissues in 1.5 ml tubes were then centrifuged at 1200 g for 2 min at room temperature (RT) and the supernatant was discarded carefully. Next, 300 µl of TrypLE (Thermo Fisher Scientific, #12604021) was added to each 1.5 ml tube and incubated at 28°C for 5 min in a thermomixer at 800 g. Once the embryos were dissociated, embryo tissues were pipetted up and down several times, followed by centrifugation at 1200 g for 4 min. Cell pellets were resuspended in 400 µl growth media containing 15% fetal bovine serum (FBS) (Cytiva, #SH30071.02E), 0.8 mM calcium chloride, 1 µg/ml human insulin solution (Sigma, #I9278), and 1X L-Glutamine (Thermo Fisher Scientific, #25030081) in L-15 (Corning, #10-045-CV), and transferred to 1 well of a 48 well plate coated with 0.1% gelatin in advance. Cells were maintained and incubated with growth media in the incubator at 28°C without CO₂ and were observed every day with media changes 3 times per week (400 µl per well). Cells usually reached confluency 7–10 days after derivation. Cells were passaged once a week at a 1:4 to 1:10 ratio and expanded for cryopreservation in freezing media (90% FBS + 10% DMSO) at 2×10^6 per vial.

Host cell reactivation (HCR) assay

Experiments were designed and protocols were modified based on two previous studies (Domankevich et al., 2018 [DOI](#); Tian et al., 2019 [DOI](#)). An amount of 25 µl of pmaxGFP plasmid from Lonza Amaxa cell Nucleofector Kit V (Lonza, #VCA-1003) at a concentration of ~0.2 µg/µl was aliquoted into a 10 cm plate and irradiated with 600 J/m² of UV-C light with the lid open. The same batch of UV-treated plasmids was used for testing all species to avoid batch-to-batch variation. Next, 1.2×10^6 cells were co-transfected with 6 µg of treated or untreated pmaxGFP. For each reaction, 82 µl base media and 18 µl of supplement was used to make the Nucleofector® Solution. Cell pellets were resuspended carefully in 100 µl RT Nucleofector® Solution per sample with 5 µg treated or untreated pmaxGFP added, then transferred into a certified cuvette. It was necessary for the samples to cover the bottom of the cuvette without air bubbles. Transfections were carried out with program T-027 using Amaxa Nucleofector II. Cuvettes were taken out of the holder once the program was finished. An amount of 20 µl of the mixture was added to 24-well plates with 1 ml of cell media and imaged every 1.5 hours for 3 days using a Nikon ECLIPSE Ti2 microscope with a 10X lens; the rest of the cells were added to 6-well plates with 3 ml of cell media for flow cytometry analysis.

Flow cytometry

Cells were harvested 50 hours post-transfection. The cell pellet collected from each well of a 6-well plate was resuspended in 500 μ l 1X PBS. An amount of 1 μ l of Ghost Dye solution (Cytek Bioscience, #13-0865-T100) was added to each 500 μ l cell suspension, vortexed immediately, and was then incubated for 30 min at 2–8°C protected from light. After incubation, the cell suspension was spun at 400 g for 5 min at RT and the supernatant was discarded. Next, 500 μ l media was added to the cell pellets to prepare for flow cytometry using Cytek Aurora. The %GFP relative expression (%RE) was calculated using $F = N \times \text{MFI}/S$, where N is the total number of live cells appearing in the positive region for GFP, MFI is the mean fluorescence intensity of the N cells, and S is the total number of live cells; $\%RE = F_t/F_u$, where F_t is the F of cells transfected with treated plasmids and F_u is the F of cells transfected with undamaged plasmids. Live cells were identified based on altered forward and side scatters.

Immunohistochemistry

For immunostaining in fish, larvae aged 6 dpf were fixed at ZT1 in a 1X Phosphate Buffer Solution (PBS) with 4% Paraformaldehyde and 0.1% Tween 20 for 6 hours on ice as previously described (Jaggard et al., 2020 [DOI](#); Kozol et al., n.d.). After fixation, samples were rinsed 3 times in PBS with 0.1% Tween 20 (PBT) at RT, with 10 min between rinses. Samples were incubated overnight at 4°C in 1:500 anti- γ H2AX (Genetex, #GTX127342) with 0.1% PBT and 2% Bovine Serum Albumin (BSA). After overnight incubation, samples were rinsed 3 times in 0.1% PBT at RT, with 10 min between rinses. Next, samples were incubated in 1:500 Goat anti-rabbit 488 (Abcam, #ab150077) and 1 μ g/ml DAPI (Thermo Fisher Scientific, #D1306) in 0.1% PBT. Finally, samples were rinsed again 3 times in 0.1% PBT at RT with 10 min between rinses, stored overnight at 4°C, and then imaged on a Nikon A1 confocal microscope with a 20X water immersion lens.

For vimentin staining in cell culture, cells were fixed with 4% Paraformaldehyde in PBS for 15 min at RT. After fixation, cells were rinsed 3 times in PBS at RT, with 5 min between rinses. After fixation, cells can be stored for up to 1 week at 4°C. Fixed cells were permeabilized with 0.1% Triton in PBS for 30 min at RT, then blocked with 20% FBS diluted in 0.1% PBT for 1 hour at RT. Samples were then incubated overnight at 4°C with 1:200 anti-vimentin antibody (Sigma, #V5255) in blocking buffer. After overnight incubation, samples were rinsed 3 times in 0.1% PBT at RT with 5 min between rinses. Next, samples were incubated for 1 hour at room temperature in darkness with 1:300 Donkey Anti-Mouse IgG (H+L) 568 (Biotium, #20105) and 1 μ g/ml DAPI (Sigma, #10236276001) in 5% FBS and 0.1% PBT. Finally, samples were rinsed again 3 times in 0.1% PBT with 5 min between rinses, rinsed once with PBS, stored overnight at 4°C, and then imaged on a Nikon ECLIPSE Ti2 confocal microscope with a 40X water immersion lens.

For CPD staining in cell culture, cells were plated at 2×10^5 per well in a glass bottom dish (Cellvis, #D35C4-20-1.5-N) 2 days before the experiment. Cells were washed once with PBS and cell media was removed before exposure to 100 J/m² UV-C (Stratalinker® UV Crosslinker, Model 2400, #400075). Cells were fixed 5 min after UV-C treatment using 4% Paraformaldehyde in PBS for 15 min at RT. After fixation, cells were rinsed 3 times in PBS at RT with 5 min between rinses. After fixation, cells can be stored up to 1 week at 4°C. Fixed cells were permeabilized with 0.1% Triton in PBS for 30 min at RT, followed by 2 rinses in PBS with 5 min between rinses. Next, 2M Hydrochloric acid (HCl) was used to denature cellular DNA for 30 min at RT, followed by 5 rinses in 0.1% PBT with 5 min between rinses. After this, cells were blocked with 20% FBS diluted in 0.1% PBT for 1 hour at RT. Samples were then incubated overnight at 4°C with 1:1000 anti-CPD antibody (TDM-2, Cosmo Bio, #CAC-NM-DND-001) in blocking buffer. After overnight incubation, samples were rinsed 3 times in 0.1% PBT at RT with 5 min between rinses. Next, samples were incubated for 1 hour in darkness with 1:300 Donkey Anti-Mouse IgG (H+L) 568 (Biotium, #20105) and 1 μ g/ml

DAPI (Sigma, #10236276001) in 5% FBS and 0.1% PBT. Finally, samples were rinsed again 3 times in 0.1% PBT with 5 min between rinses, rinsed once with PBS, stored overnight at 4°C, and then imaged on a Nikon ECLIPSE Ti2 confocal microscope with a 40X water immersion lens.

Western Blot analysis

Fish fibroblast cells and liver-derived cells (Krishnan et al., 2022 [DOI](#)) were plated at 5×10^4 per well in 6-well plates 4 days before the experiment. Cell media was removed before cells were exposed to 3000 J/m² UV-C. Fresh cell growth media was added to the cells and incubated for 2, 4, or 6 hours. Lysis buffer was made with 1X protease inhibitor (25X stock, Sigma, #11873580001) and 1X phosphatase inhibitor (100X stock, Cell Signaling, #5870S) in RIPA buffer (Thermo Fisher Scientific, #89900). Next, 100 µl lysis buffer was added to each well and cells were detached by cell scraper (VWR INTERNATIONAL, #10062-904). Samples were fully lysed by pipetting up and down ~5 times with a fine needle syringe (BD Medical, #328438) and vortexed for 10 seconds. The supernatant was collected as protein fractions after two rounds of centrifugation at 12,000 g for 20 min at 4°C. Protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, #23227).

Protein samples were loaded to the protein gel (Thermo Fisher Scientific, #NP0323BOX) at ~10–13 µg per lane. Protein gels were run in MOPS buffer (Thermo Fisher Scientific, #NP0001) at 130 V for 80 min, then transferred to PVDF membrane (Sigma, #IPVH00010) in 4°C at 215 mA for 50 min. PVDF membranes were blocked in blocking buffer (LI-COR, #927-80001) for an hour at RT, then incubated with primary antibody overnight at 4°C in 1:1000 anti-γH2AX (Genetex, #GTX127342) and 1:1000 anti-GAPDH (Proteintech, #60004-1-Ig) with blocking buffer. On the next day, PVDF membranes were washed 3 times in 0.1% TBST with 5 min between rinses and incubated with 1:10,000 Donkey anti-mouse (LI-COR, #926-32212) and anti-rabbit (LI-COR, #926-68073) secondary antibody at RT for an hour. Finally, samples were rinsed again 3 times in 0.1% TBST with 10 min between rinses, and then imaged and quantified on LI-COR Odyssey® CLx with Image Studio v5.2 software.

ROS imaging

ROS imaging was implemented based on previous protocols used in *Drosophila* (Vaccaro et al., 2020 [DOI](#)). Larvae aged 6 dpf were euthanized at ZT1 by immersion in ice-chilled aquarium water for 30 min. Following euthanasia, samples were incubated in 60 µM dihydroethidium (DHE; Thermo Fisher Scientific, D11347) for 5 min at RT, covered in foil to protect it from light. Next, samples were rinsed for 5 min in 1X PBS, then mounted in 2% low melting point agarose (Sigma-Aldrich, A9414) and the entire gut was imaged on a Nikon A1R confocal microscope with a 10X air objective, with a slice thickness of 4 µm. After interaction with superoxide radicals, DHE is converted into 2-hydroxy ethidium, which is optimally excited at 480 nm and fluoresces red (wavelength > 550 nm); therefore, the sample was excited with a 488 nm laser and imaged with a detector in the 570–616 nm range (Kumar and R. Gullapalli, 2024). ROS in the gut was quantified by calculating a z-projection (calc: Average Intensity) in ImageJ (NIH, v1.54f) and manually drawing ROIs around anatomically defined regions of the gut (Riddle et al., 2018b [DOI](#)).

Sleep experiments

Sleep experiments used previously described methodology (Jaggard et al., 2019 [DOI](#)). For induction of DNA damage, larvae aged 5 dpf were transferred to individual wells of a 24-well plate at ZT7–8 (VWR, 82050-892), and acclimated overnight to the testing environment. At ZT0 the next day, larvae were subjected to either 60 seconds of control white light or 30 or 60 seconds of UV-B light in a UV-Crosslinker cabinet (Spectro-UV, XL-1500) fitted with UV bulbs with a spectral peak at 306 nm and a power output of 32.3 µW/cm² at 1 meter. (UV-B; Spectro-UV, BLE-1T158). Following treatment, well plates were immediately returned to the testing environment and filmed from above using a USB camera (Basler, acA1300-200) fitted with a 16 mm fixed focal length lens

(Edmund Optics, 67-714) and an infrared pass filter (Edmund Optics, 65-796) to ensure consistent image quality after the day/night transition. Larvae were lit from below using infrared light strips (850 nm), diffused through a custom-made white acrylic light box (TAP Plastics).

Locomotor behavior was tracked in Ethovision XT (Noldus), and frame-by-frame velocity data was exported and analyzed using a custom Python script, with sleep defined as 60 seconds or more of consolidated immobility. A velocity cut-off of 6 mm/s was used to distinguish active swimming from passive drift.

RNA extraction and sequencing

For RNA sequencing on fish fibroblast cells, cells were detached with TrypLE and washed once with cell media. Cell pellets were collected after centrifugation at 1000 g for 5 min, snap frozen in liquid nitrogen, and stored at -80°C prior to RNA extraction. For UV RNA sequencing experiments, larvae were treated identically to the sleep experiments until 1.5 hours after UV exposure, at which point they were transferred to 1.5 ml centrifuge tubes, with 4 larvae pooled into each tube. The larvae were euthanized by chilling on ice before immediately proceeding to RNA extraction.

For RNA sequencing experiments on adults, dissections were performed at Florida Atlantic University. Briefly, tissue was dissected between ZT0 and ZT3 from at least 4 fish at each age and population group. Tissue was immediately flash frozen in liquid nitrogen. To extract RNA, 1 ml of TRIzol was added to each sample. Samples were homogenized, and then 200 µl chloroform was added to each tube, followed by vigorous shaking. Samples were incubated on ice for 15 min, then phase separated by centrifuging at 12,000 g for 15 min at 4°C. The resulting aqueous phase of the liquid was transferred to a fresh 1.5 ml tube, and then RNA was precipitated out by mixing with 0.5 ml isopropanol. Samples were incubated on ice for 10 min, then centrifuged at 12,000 g for 10 min at 4°C. The supernatant was removed by pouring and lightly shaking the tube, then the resulting pellet was washed by the addition of 1 ml 70% EtOH while vigorously flicking the tube. The samples were then centrifuged at 7500 g for 10 min at 4°C. The supernatant was removed by pouring and lightly shaking the tube, and the samples were air dried upside down for 10 min. Finally, the pellet was redissolved in 100 µl of RNase-free H₂O.

RNA sequencing data analysis

RNA sequencing for the UV and Aging experiments were performed by Novogene, on an Illumina NovaSeq X Plus. Sample quality control, library preparation, and sequencing were performed by Novogene. Raw reads received from Novogene were mapped against the *Astyanax mexicanus* reference genome (version 2.0, GenBank Accession Number: GCA_000372685.2) using the splice-aware mapper STAR (Dobin et al., 2013 [DOI](#)) to generate raw counts. Annotations were extracted from the *A. mexicanus* annotation file from Ensembl (*Astyanax_mexicanus*-2.0.108.gtf). Subsequent analysis was performed in RStudio (v4.3.0) using the differential expression testing software DESeq2 (v1.40.2) (Love et al., 2014 [DOI](#)). RNA sequencing for the fibroblast cell lines was performed on an Illumina NextSeq 500.

Data from the UV-B experiment and the aging experiment were analyzed using the same analysis pipeline, with each tissue type from the aging experiment analyzed separately. First, a DESeq object was created from the raw counts matrix and processed using the DESeq() command, which estimates size factors and dispersion, and fits the data to a negative binomial GLM. Normalized count values used in downstream analysis were generated using the counts() function (normalized = T). Sample variability for each group was visualized by first performing a variance stabilizing transformation using the vst() function, then generating a PCA plot from the resulting object with the plotPCA() function. To identify genes which were differentially expressed in response to UV-B treatment (or aging), each population treatment subset was reanalyzed as above, so that the effects of treatment on each population were considered separately. An adjusted *p*-value < 0.05 was used to determine a significant response to treatment.

Gene Ontology (GO) analysis

Gene ontology pathway analyses were performed using the clusterProfiler package (v4.8.3) in R. For gene set enrichment analyses, genes were first ranked according to the magnitude of their response; the ranking value was calculated as $-\log_{10}(\text{pval})/\text{sign}(\log_2\text{FC})$, to account for both direction and magnitude of response. The resulting ranked list was processed using the gseGO function (options: ont='BP', keyType='SYMBOL', pvalueCutoff=0.05, OrgDB = org.Dr.eG.db, pAdjustMethod = 'BH'). For visualization purposes, similar GO terms were grouped together using the simplify() function (options:by='p.adjust', select_fun=min), and visualized using the dotplot() function. For the UV-B experiment, since there were many overlapping GO terms, the top 10 unique terms in each direction were plotted.

Quantitative PCR

Adult fins were collected at Zeitgeber or Circadian time 8. 5 dpf larvae were collected at Zeitgeber or Circadian time 2, 6, 10 and 14. Samples were then homogenized and total RNA extracted as above. cDNA was synthesized from 500 ng RNA using iScript cDNA synthesis kit (BIO-RAD, #1708891). Approximately 50 ng of cDNA was used for quantitative PCR (qPCR) using Perfecta SYBR Green with Low ROX (Quantabio, #95074-012) with a QuantStudio 5 Real-Time PCR System. Specificity of each amplicon and cDNA final concentration was optimized via analysis of post-reaction dissociation curves, validating a single amplicon for each set of primers. Analysis was conducted using the $\Delta\Delta\text{Ct}$ method. All samples were run in 3-4 replicates and normalized to the housekeeping gene *rpl13a*. Primer sequences used are as follows:

cdp (ENSAMXG00000001885): FW: 5'- GGCCTCTCCTAAGCTGGAGT -3'

RV: 5'- GTCCACAGGTGGGAATTCAG -3'

ddb2 (ENSAMXG00000000525):

FW: 5'- AAGCTGCACAAAGCCAAAGT-3'

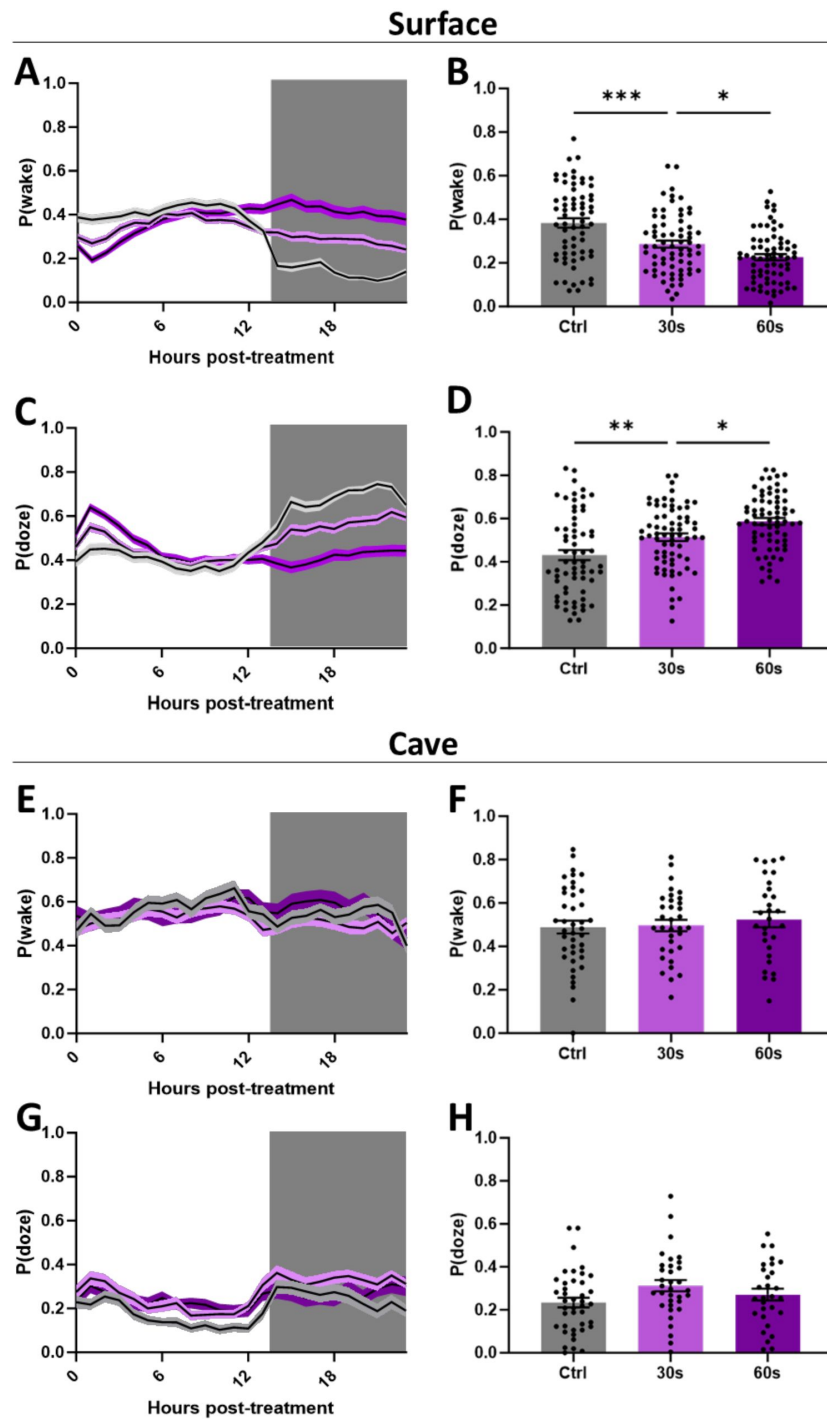
RV: 5'- AGACGATGTTGCCACTAGCC -3'.

rpl13a (ENSAMXG000000033532):

FW 5'- CGCAACAAATTGAAGTACCTG -3'

RV: 5'- GGTTCGTGTTTCATCCTCTTG -3'

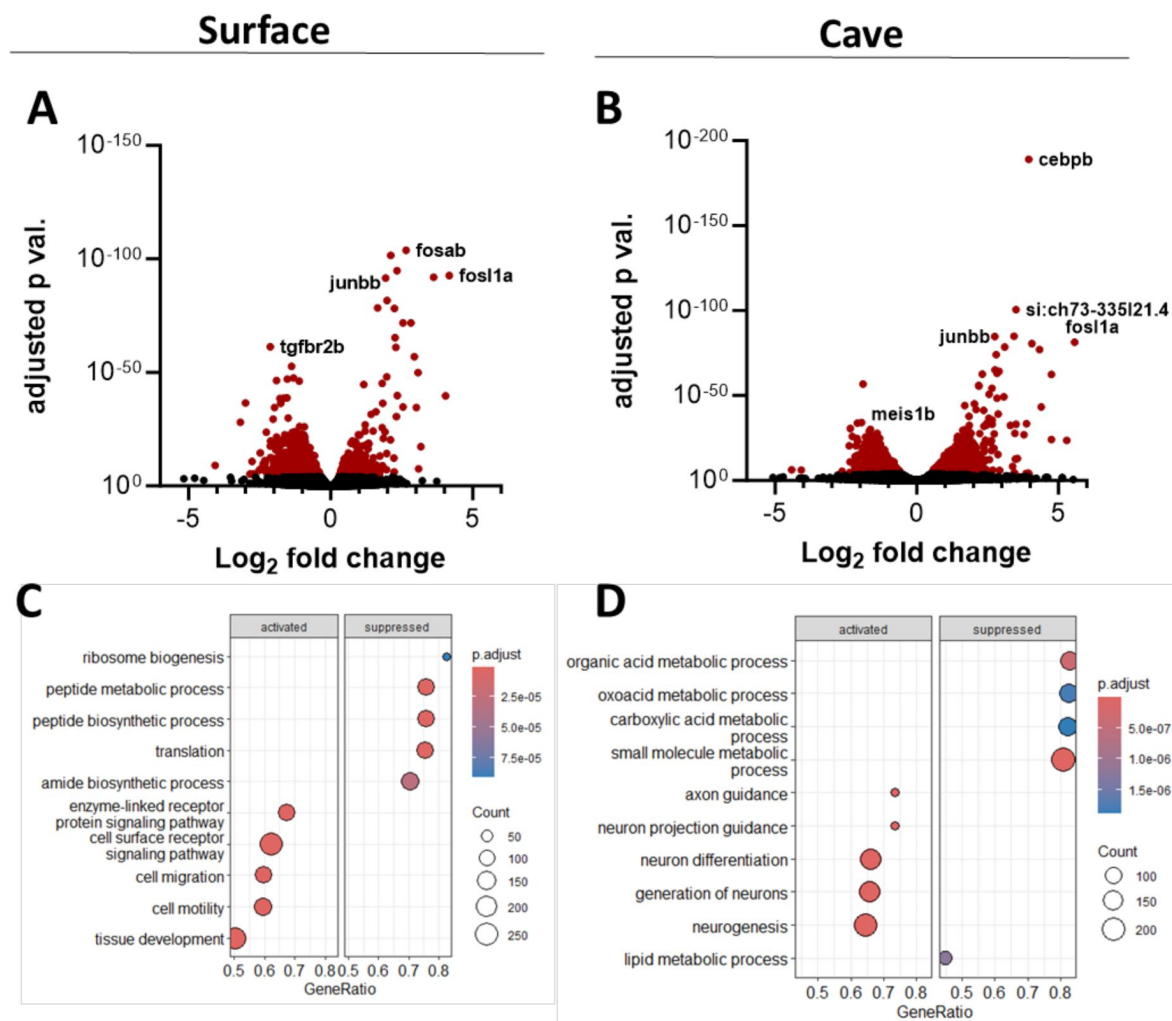
Supplementary figures



Supplemental Figure 1.

Response in sleep pressure to UV-B-induced DNA damage.

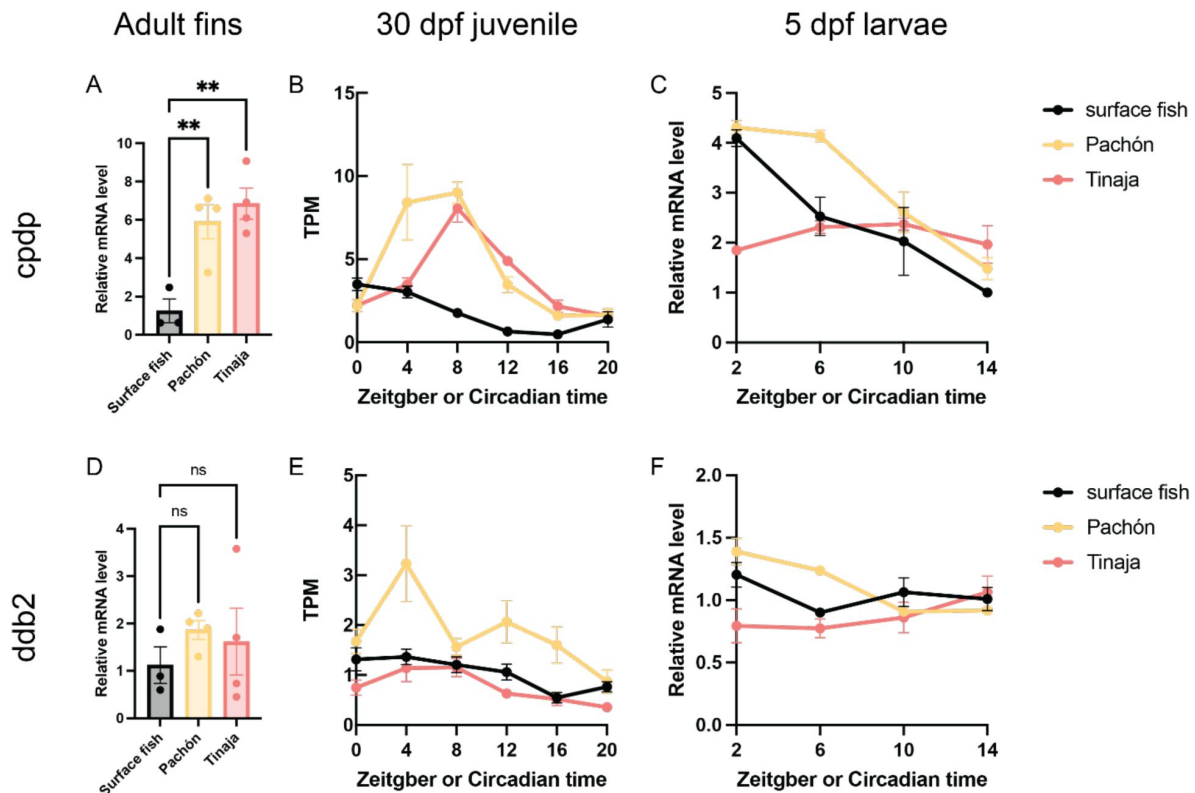
(**A**) The 24-hour $P(\text{wake})$ profiles of surface fish exposed to 30 or 60 seconds of UV-B light compared to controls. (**B**) $P(\text{wake})$ in surface fish in the 3 hours following UV-B exposure (one-way ANOVA: $F_{2, 202} = 21.08, p < 0.0001$). (**C**) The 24-hour $P(\text{doze})$ profiles of surface fish exposed to 30 or 60 seconds of UV-B light compared to controls. (**D**) $P(\text{doze})$ in surface fish in the 3 hours following UV-B exposure (one-way ANOVA: $F_{2, 202} = 14.09, p < 0.0001$). (**E**) The 24-hour $P(\text{wake})$ profiles of cavefish exposed to 30 or 60 seconds of UV-B light compared to controls. (**F**) $P(\text{wake})$ in cavefish in the 3 hours following UV-B exposure. (**G**) The 24-hour $P(\text{doze})$ profiles of cavefish exposed to 30 or 60 seconds of UV-B light compared to controls. (**H**) $P(\text{doze})$ in cavefish in the 3 hours following UV-B exposure. (ZT=Zeitgeber time). All treatments performed at ZT0.



Supplemental Figure 2.

Transcriptional responses to UV-B-induced DNA damage in surface fish and cavefish.

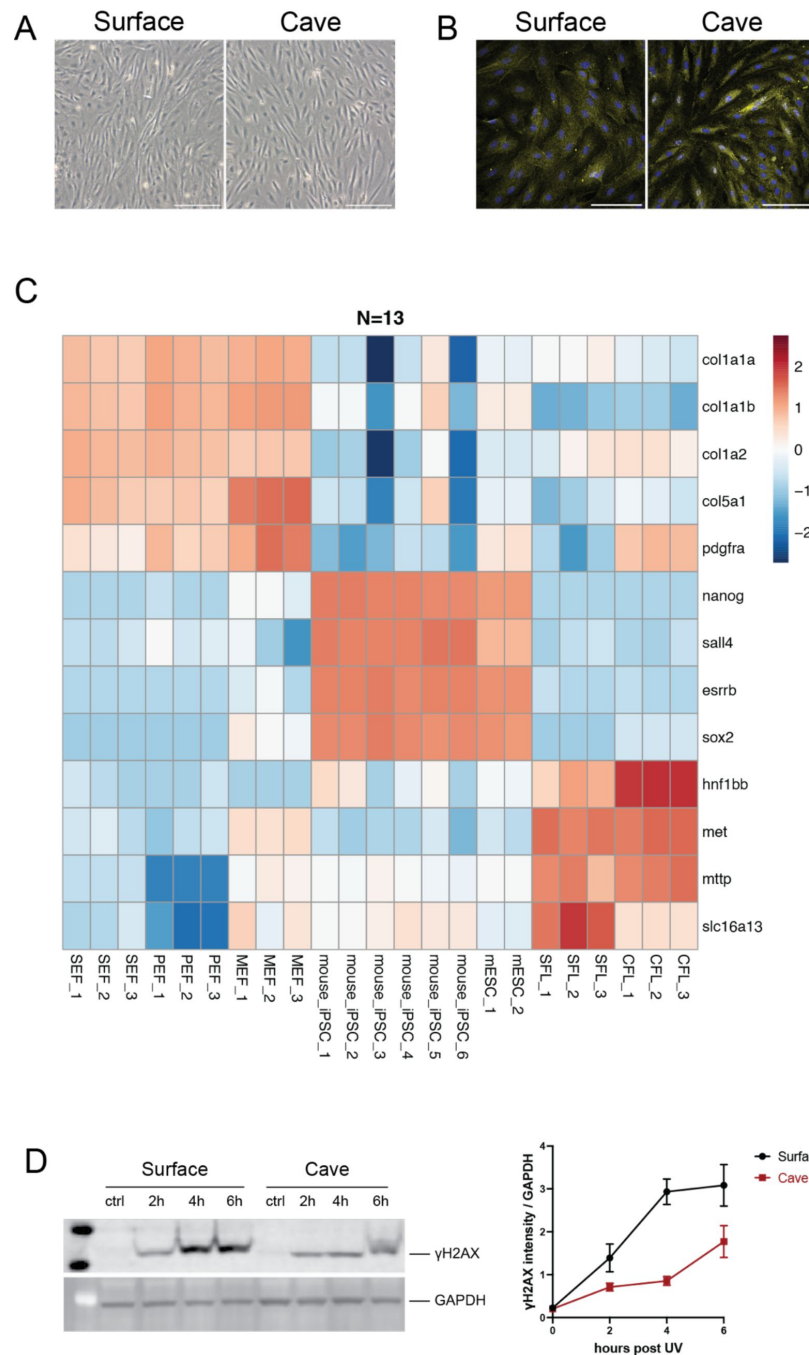
(A) Volcano plot of gene expression in surface fish in response to UV-B radiation, with significant genes highlighted in red (adjusted $p < 0.05$). (B) Volcano plot of gene expression in cavefish in response to UV-B radiation, with significant genes highlighted in red (adjusted $p < 0.05$). (C) Most highly enriched GO terms in a gene set enrichment analysis of surface fish exposed to UV-B radiation. (D) Most highly enriched GO terms in a gene set enrichment analysis of cavefish exposed to UV-B radiation.



Supplemental Figure 3.

DNA repair gene expression in surface fish and cavefish.

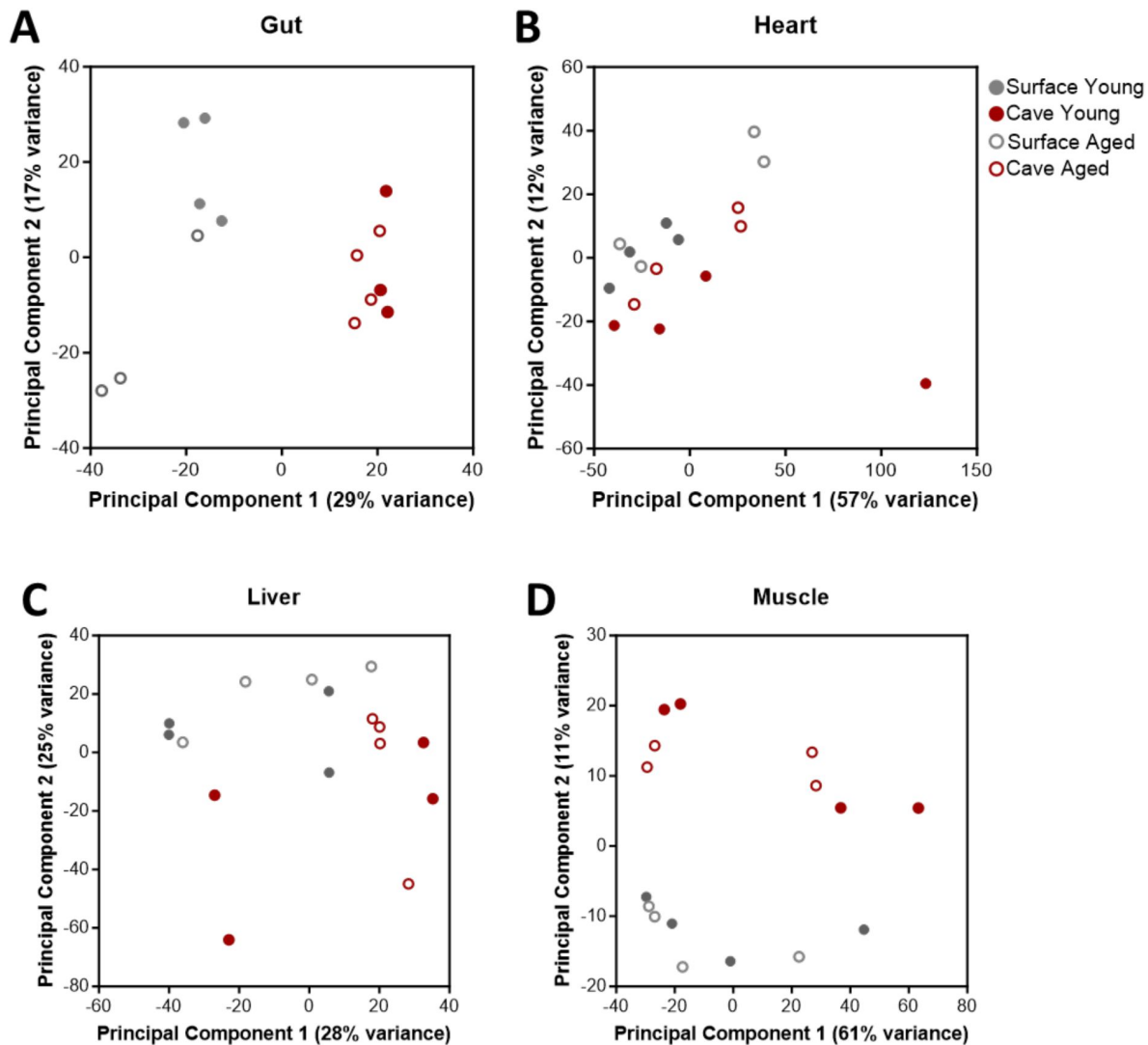
(A,D) Adult fins were collected at Zeitgeber or Circadian time 8. *cpdp* (A) and *ddb2* (D) mRNA levels were determined by qPCR. Expression of *cpdp* (one-way ANOVA: $F = 11.99$, $p = 0.0039$) and *ddb2* (one-way ANOVA: $F = 0.7576$, $p = 0.4997$) was compared among all populations. ns $p \geq 0.05$, ** $p < 0.01$. (B,E) RNA sequencing data obtained from *cpdp* (B) and *ddb2* (E) gene expression level in 30 dpf juvenile fish. (TPM: transcript per million). (C,F) Fish larvae aged 5 dpf were collected at different Zeitgeber or Circadian times. *cpdp* (C) and *ddb2* (F) mRNA levels were determined by qPCR.



Supplemental Figure 4.

Pachón cavefish-derived cells exhibit a lower UV-induced DNA damage response and repair compared to surface fish.

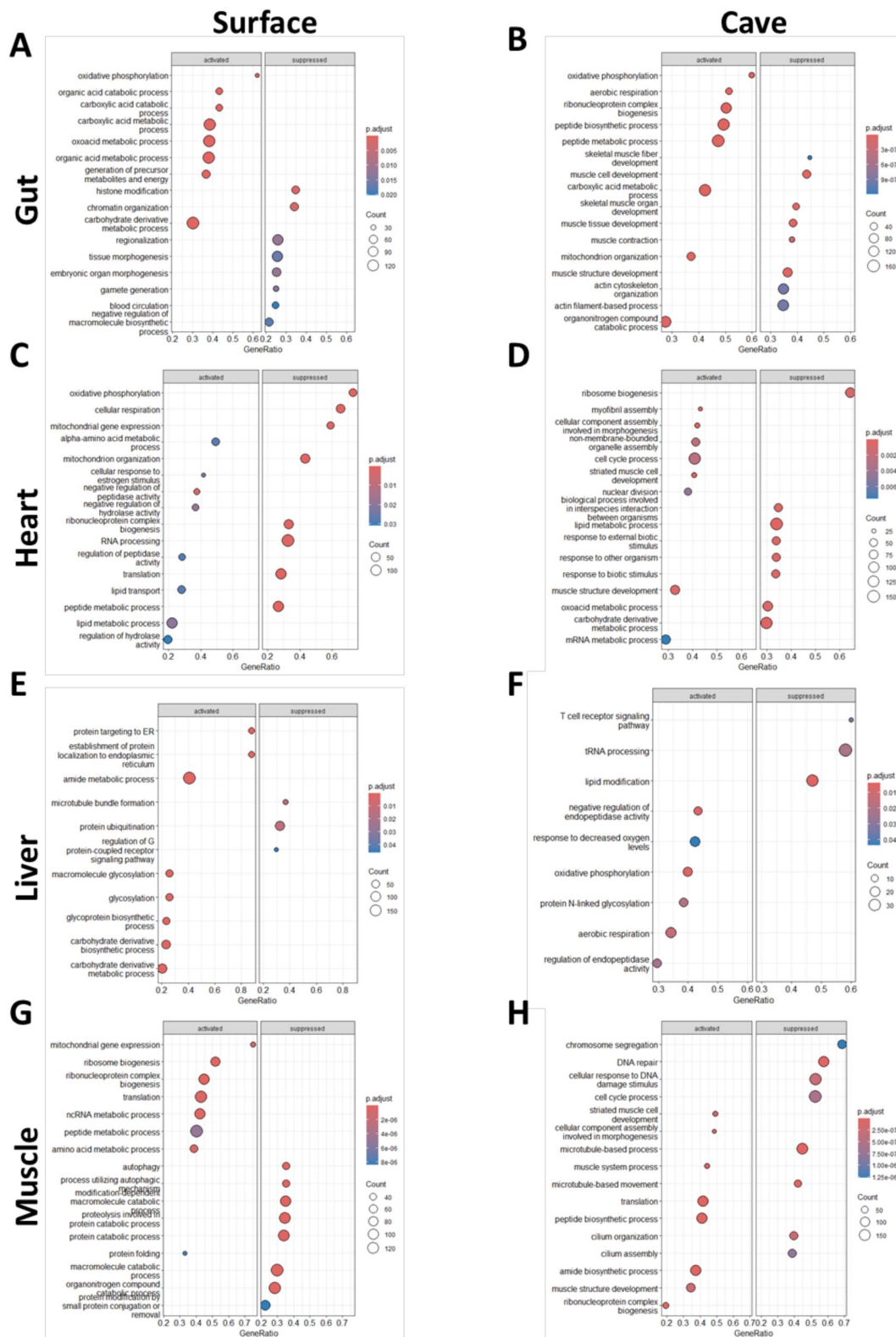
(A) Bright field images of surface fish and Pachón cavefish embryonic fibroblasts. Scale bar, 200 μ m. **(B)** Immunostaining of vimentin in surface fish and Pachón cavefish embryonic fibroblasts. Yellow and blue show vimentin and DAPI staining, respectively. Scale bar, 100 μ m. **(C)** Heat map of differentially expressed genes among different cell types. The number below the heat map indicates independent biological replicates ($n = 13$). Red and blue indicate upregulated and downregulated genes, respectively. (SEF: surface fish embryonic fibroblast; PEF: Pachón cavefish embryonic fibroblast; MEF: mouse embryonic fibroblast; iPSC: induced pluripotent stem cell; mESC: mouse embryonic fibroblast; SFL: surface fish liver-derived cell; CFL: Pachón cavefish liver-derived cell). **(D)** Western blot of γ H2AX indicates a diminished DNA damage response in Pachón cavefish liver-derived cells compared to surface fish liver-derived cells.



Supplemental Figure 5.

Aging-induced changes in gene expression.

Multi-dimensional scaling plots of gene expression in young and aged surface fish and cavefish samples, in the gut **(A)** PC1 (two-way ANOVA: (Treatment) $F_{1,11}=10.41$, $p=0.0081$, (Population) $F_{1,11}=264$, $p<0.0001$, (Interaction) $F_{1,11}=3.135$, $p=0.1043$. PC2 (two-way ANOVA: (Treatment) $F_{1,11}=8.257$, $p=0.0151$, (Population) $F_{1,11}=0.594$, $p=0.4571$, (Interaction) $F_{1,11}=7.113$, $p=0.0219$. Heart **(B)** PC1 (two-way ANOVA: (Treatment) $F_{1,12}=0.0302$, $p=0.8649$, (Population) $F_{1,12}=0.8455$, $p=0.376$, (Interaction) $F_{1,12}=0.9535$, $p=0.3481$. PC2 (two-way ANOVA: (Treatment) $F_{1,12}=7.354$, $p=0.0189$, (Population) $F_{1,12}=0.7584$, $p=0.0175$, (Interaction) $F_{1,12}=0.3345$, $p=0.5737$. Liver **(C)** PC1 (two-way ANOVA: (Treatment) $F_{1,12}=1.067$, $p=0.3219$, (Population) $F_{1,12}=4.521$, $p=0.0549$, (Interaction) $F_{1,12}=0.1329$, $p=0.7217$. PC2 (two-way ANOVA: (Treatment) $F_{1,12}=2.039$, $p=0.1788$, (Population) $F_{1,12}=6.986$, $p=0.0214$, (Interaction) $F_{1,12}=0.0419$, $p=0.8413$. Muscle **(D)** PC1 (two-way ANOVA: (Treatment) $F_{1,12}=0.5895$, $p=0.4574$, (Population) $F_{1,12}=0.7313$, $p=0.4092$, (Interaction) $F_{1,12}=0.01396$, $p=0.9079$. PC2 (two-way ANOVA: (Treatment) $F_{1,12}=0.15$, $p=0.7053$, (Population) $F_{1,12}=89.42$, $p<0.0001$, (Interaction) $F_{1,12}=0.009$, $p=0.9251$.



Supplemental Figure 6.

GSEA analyses of aging surface fish and cavefish tissues.

Top GO terms resulting from GSEA analysis in aged surface fish (left) and cavefish (right) tissues. Gut (**A,B**), heart (**C,D**), liver (**E,F**), and muscle (**G,H**). Top 8 results in each direction are shown; if less than 8 terms were enriched, all results are shown.

Data availability

Raw data from RNASeq experiments have been deposited in GEO as a SuperSeries, under accession code GSE272697. Data from UV-B experiments has been deposited under accession code GSE271729. Data from aging experiments has been deposited under accession code GSE272611. Raw count data, and results of differential gene expression analysis, have been uploaded as supplemental data 1 (UV) and supplemental data 2 (aging).

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Author information

Evan Lloyd*

Department of Biology, Texas A&M University, College Station, United States

*Denotes equal contributions

Fanning Xia*

Stowers Institute for Medical Research, Kansas City, United States

*Denotes equal contributions

Kinsley Moore

Department of Biology, Texas A&M University, College Station, United States

Carolina Zertuche

Department of Biology, Texas A&M University, College Station, United States

Aakriti Rastogi

Department of Biology, Texas A&M University, College Station, United States

Rob Kozol

Harriet Wilkes Honors College, Florida Atlantic University, Jupiter, United States

Olga Kenzior

Stowers Institute for Medical Research, Kansas City, United States

Wesley Warren

Department of Genomics, University of Missouri, Columbia, United States

Lior Appelbaum

Faculty of Life Science and the Multidisciplinary Brain Research Center, Bar Ilan University, Ramat Gan, Israel

Rachel L Moran

Department of Biology, Texas A&M University, College Station, United States

Chongbei Zhao

Stowers Institute for Medical Research, Kansas City, United States

Erik Duboue

Harriet Wilkes Honors College, Florida Atlantic University, Jupiter, United States

Nicolas Rohner

Stowers Institute for Medical Research, Kansas City, United States

For correspondence: nro@stowers.org

Alex C Keene

Department of Biology, Texas A&M University, College Station, United States

For correspondence: keenea@tamu.edu

Editors

Reviewing Editor

Filippo Del Bene

Institut de la Vision, Paris, France

Senior Editor

Claude Desplan

New York University, New York, United States of America

Reviewer #1 (Public review):

Summary:

Lloyd et al employ an evolutionary comparative approach to study how sleep deprivation affects DNA damage repair in *Astyanax mexicanus*, using the cave vs surface species evolution as a playground. The work shows, convincingly, that the cavefish population has evolved an impaired DNA damage response both following sleep deprivation or a classical paradigm of DNA damage (UV).

Strengths:

The study employs a thorough multidisciplinary approach. The experiments are well conducted and generally well presented.

Weaknesses:

Having a second experimental mean to induce DNA damage would strengthen and generalise the findings.

Overall, the study represents a very important addition to the field. The model employed underlines once more the importance of using an evolutionary approach to study sleep and provides context and caveats to statements that perhaps were taken a bit too much for granted before. At the same time, the paper manages to have an extremely constructive approach, presenting the platform as a clear useful tool to explore the molecular aspects behind sleep and cellular damage in general. The discussion is fair, highlighting the strengths and weaknesses of the work and its implications.

Comments on revisions:

I was pretty happy with the previous version of the manuscript already and the authors have made all the minor corrections I had suggested so I don't have much to add. The main "weakness", if at all, is that the story would benefit from a secondary stressor (other than UV) but I understand the authors see this more as a long term development than just an addition to this particular paper, which is fair enough.

I don't have any further recommendations. I think this model system is really important for the sleep field and offers a completely new and important perspective to its evolution and function.

<https://doi.org/10.7554/eLife.99191.2.sa3>

Reviewer #2 (Public review):

The manuscript investigates the relationship between sleep, DNA damage, and aging in the Mexican cavefish (*Astyanax mexicanus*), a species that exhibits significant differences in sleep patterns between surface-dwelling and cave-dwelling populations. The authors aim to understand whether these evolved sleep differences influence the DNA damage response (DDR) and oxidative stress levels in the brain and gut of the fish.

Summary of the Study:

The primary objective of the study is to determine if the reduced sleep observed in cave-dwelling populations is associated with increased DNA damage and altered DDR. The authors compared levels of DNA damage markers and oxidative stress in the brains and guts of surface and cavefish. They also analyzed the transcriptional response to UV-induced DNA damage and evaluated the DDR in embryonic fibroblast cell lines derived from both populations.

Strengths of the Study:

Comparative Approach: The study leverages the unique evolutionary divergence between surface and cave populations of *A. mexicanus* to explore fundamental biological questions about sleep and DNA repair.

Multifaceted Methodology: The authors employ a variety of methods, including immunohistochemistry, RNA sequencing, and in vitro cell line experiments, providing a comprehensive examination of DDR and oxidative stress.

Interesting Findings: The study presents intriguing results showing elevated DNA damage markers in cavefish brains and increased oxidative stress in cavefish guts, alongside a reduced transcriptional response to UV-induced DNA damage.

Weaknesses of the Study:

Link to Sleep Physiology: The evidence connecting the observed differences in DNA damage and DDR directly to sleep physiology is not convincingly established. While the study shows distinct DDR patterns, it does not robustly demonstrate that these are a direct result of sleep differences.

Causal Directionality: The study fails to establish a clear causal relationship between sleep and DNA damage. It is possible that both sleep patterns and DDR responses are downstream effects of a common cause or independent adaptations to the cave environment.

Environmental Considerations: The lab conditions may not fully replicate the natural environments of the cavefish, potentially influencing the results. The impact of these conditions on the study's findings needs further consideration.

Photoreactivity in Albino Fish: The use of UV-induced DNA damage as a primary stressor may not be entirely appropriate for albino, blind cavefish. Alternative sources of genotoxic stress should be explored to validate the findings.

Assessment of the Study's Achievements:

The authors partially achieve their aims by demonstrating differences in DNA damage and DDR between surface and cavefish. However, the results do not conclusively support the claim that these differences are driven by or directly related to the evolved sleep patterns in cavefish. The study's primary claims are only partially supported by the data.

Impact and Utility:

The findings contribute valuable insights into the relationship between sleep and DNA repair mechanisms, highlighting potential areas of resilience to DNA damage in cavefish. While the direct link to sleep physiology remains unsubstantiated, the study's data and methods will be useful to researchers investigating evolutionary biology, stress resilience, and the molecular basis of sleep.

Comments on revisions:

The manuscript should tone down claims of a direct causal relationship between sleep differences and DDR outcomes, acknowledging the possibility that both are independent or downstream adaptations to the cave environment. To strengthen the study, the authors should adopt additional genotoxic stressors, such as chemical agents (e.g., cisplatin or hydrogen peroxide) or physical stress (e.g., ionizing radiation), to validate findings beyond UV-induced DNA damage, which may not be ideal for albino cavefish. Explicitly discussing the influence of laboratory conditions, such as water quality, lighting, and diet, on oxidative stress and DDR phenotypes, and comparing lab-reared and wild-caught fish if feasible, would bolster ecological relevance. The study should clarify that the current data do not establish a causal link between sleep and DNA damage, instead proposing this as a hypothesis for future research. Expanding the evolutionary context by linking DDR differences to other cavefish traits, such as metabolic efficiency or hypoxia tolerance, could provide a more integrative perspective. Additionally, proposing future experiments involving pharmacological or behavioral manipulation of sleep, as well as incorporating comparative genomics or transcriptomics to identify DDR-related genetic adaptations, would enhance the study's depth.

<https://doi.org/10.7554/eLife.99191.2.sa2>

Reviewer #3 (Public review):

Lloyd, Xia et al. utilised the existence of surface-dwelling and cave-dwelling morphs of *Astyanax mexicanus* to explore a proposed link between DNA damage, aging, and the evolution of sleep. Key to this exploration is the behavioural and physiological differences between cavefish and surface fish, with cavefish having been previously shown to have low levels of sleep behaviour, along with metabolic alterations (for example chronically elevated blood glucose levels) in comparison to fish from surface populations. Sleep deprivation, metabolic dysfunction and DNA damage are thought to be linked, and to all contribute to aging processes. Given that cavefish seem to show no apparent health consequences of low sleep levels, the authors suggest that they have evolved resilience to sleep loss. Furthermore, as extended wake and loss of sleep is associated with increased rates of damage to DNA (mainly double-strand breaks) and sleep is linked to repair of damaged DNA, the authors propose that changes in DNA damage and repair might underlie the reduced need for sleep in the cavefish morphs relative to their surface-dwelling conspecifics.

To fulfil their aim of exploring links between DNA damage, aging, and the evolution of sleep, the authors employ methods that are largely appropriate, and comparison of cavefish and surface fish morphs from the same species certainly provides a lens by which cellular, physiological and behavioural adaptations can be interrogated. Fluorescence and

immunofluorescence are used to measure gut reactive oxygen species and markers of DNA damage and repair processes in the different fish morphs, and measurements of gene expression and protein levels are appropriately used. However, although the sleep tracking and quantification employed is quite well established, issues with the experimental design relating to attempts to link induced DNA damage to sleep regulation (outlined below). Moreover, although the methods used are appropriate for the study of the questions at hand, there are issues with the interpretation of the data and with these results being over-interpreted as evidence to support the paper's conclusions.

This study shows that a marker of DNA repair molecular machinery that is recruited to DNA double-strand breaks (γ H2AX) is elevated in brain cells of the cavefish relative to the surface fish, and that reactive oxygen species are higher in most areas of the digestive tract of the cavefish than in that of the surface fish. As sleep deprivation has been previously linked to increases in both these parameters in other organisms (both vertebrates and invertebrates), their elevation in the cavefish morph is taken to indicate that the cavefish show signs of the physiological effects of chronic sleep deprivation.

It has been suggested that induction of DNA damage can directly drive sleep behaviour, with a notable study describing both the induction of DNA damage and an increase in sleep/immobility in zebrafish (*Danio rerio*) larvae by exposure to UV radiation (Zada et al. 2021 doi:10.1016/j.molcel.2021.10.026). In the present study, an increase in sleep/immobility is induced in surface fish larvae by exposure to UV light, but there is no effect on behaviour in cavefish larvae. This finding is interpreted as representing a loss of a sleep-promoting response to DNA damage in the cavefish morph. However, induction of DNA damage is not measured in this experiment, so it is not certain if similar levels of DNA damage are induced in each group of intact larvae, nor how the amount of damage induced compares to the pre-existing levels of DNA damage in the cavefish versus the surface fish larvae. In both this study with *A. mexicanus* surface morphs and the previous experiments from Zada et al. in zebrafish, observed increases in immobility following UV radiation exposure are interpreted as following from UV-induced DNA damage. However, in interpreting these experiments it is important to note that the cavefish morphs are eyeless and blind. Intense UV radiation is aversive to fish, and it has previously been shown in zebrafish larvae that (at least some) behavioural responses to UV exposure depend on the presence of an intact retina and UV-sensitive cone photoreceptors (Guggiana-Nilo and Engert, 2016, doi:10.3389/fnbeh.2016.00160). It is premature to conclude that the lack of behavioural response to UV exposure in the cavefish is due to a difference response to DNA damage, as their lack of eyes will likely inhibit a response to the UV stimulus. Indeed, were the equivalent zebrafish experiment from Zada et al. to be repeated with mutant larvae fish lacking the retinal basis for UV detection it might be found that, in this case too, the effects of UV on behaviour are dependent on visual function. Such a finding should prompt a reappraisal of the interpretation that UV exposure's effects on fish sleep/locomotor behaviour are mediated by DNA damage. An additional note, relating to both Lloyd, Xia et al. and Zada et al., is that though increases in immobility are induced following UV exposure, in neither study have assays of sensory responsiveness been performed during this period. As a decrease in sensory responsiveness is a key behavioural criterion for defining sleep, it is therefore unclear that this post-UV behaviour is genuinely increased sleep as opposed to a stress-linked suppression of locomotion due to the intensely aversive UV stimulus. While it is true that behavioural immobility is used by many studies as a criterion to identify sleep in non-mammalian species, this is only fully appropriate when other elements of the behavioural criteria of sleep (e.g. reduced responsiveness to sensory stimuli, rapid reversibility, homeostatic regulation, circadian regulation) have been shown to be associated with these periods of behavioural quiescence. In both Lloyd, Xia et al. and Zada et al., only an increased immobility has been demonstrated, occurring at a period where the circadian clock would be promoting wake and natural homeostatic sleep drive would be expected to be at the low end of its normal range. At a minimum, testing sensory threshold would be advisable to

ensure that the classification of this behaviour as sleep is accurate and to avoid the risk of being misled in the interpretation of these experiments.

The effects of UV exposure, in terms of causing damage to DNA, inducing DNA damage response and repair mechanisms, and in causing broader changes in gene expression are assessed in both surface and cavefish larvae, as well as in cell lines derived from these different morphs. Differences in the suite of DNA damage response mechanisms that are upregulated are shown to exist between surface fish and cavefish larvae, though at least some of this difference is likely to be due to differences gene expression that may exist even without UV exposure (this is discussed further below).

UV exposure induced DNA damage (as measured by levels of cyclobutene pyrimidine dimers) to a similar degree in cell lines derived from both surface fish and cave fish. However, γ H2AX shows increased expression only in cells from the surface fish, suggesting an induction of an increased DNA repair response in these surface morphs, corroborated by their cells' increased ability to repair damaged DNA constructs experimentally introduced to the cells in a subsequent experiment. This "host cell reactivation assay" is a very interesting assay for measuring DNA repair in cell lines, but the power of this approach might be enhanced by introducing these DNA constructs into larval neurons *in vivo* (perhaps by electroporation) and by tracking DNA repair in living animals. Indeed, in such a preparation, the relationship between DNA repair and sleep/wake state could be assayed.

Comparing gene expression in tissues from young (here 1 year) and older (here 7-8 years) fish from both cavefish and surface fish morphs, the authors found that there are significant differences in the transcriptional profiles in brain and gut between young and old surface fish, but that for cavefish being 1 year old versus being 7-8 years old did not have a major effect on transcriptional profile. The authors take this as suggesting that there is a reduced transcriptional change occurring during aging and that the transcriptome of the cavefish is resistant to age-linked changes. This seems to be only one of the equally plausible interpretations of the results; it could also be the case that alterations in metabolic cellular and molecular mechanisms, and particularly in responses to DNA damage, in the cavefish mean that these fish adopt their "aged" transcriptome within the first year of life. This would mean that rather than the findings revealing that "the transcriptome of the cavefish is resilient to age-associated changes despite sleep loss, elevated ROS and elevated DNA damage", it would suggest that the cavefish transcriptome is sensitive to age-associated changes, potentially being driven by this low level of sleep, elevated reactive oxygen species, and elevated DNA damage. This alternative interpretation greatly changes the understanding of the present findings. One way in which the more correct interpretation could be determined would be by adding a further, younger group of fish to the comparison (perhaps a group in the age range of 1-3 months, relatively shortly after metamorphosis).

A major weakness of the study in its current form is the absence of sleep deprivation experiments to assay the effects of sleep loss on the cellular and molecular parameters in question. Without such experiments, the supposed link of sleep to the molecular, cellular and "aging" phenotypes remains tenuous. Although the argument might be made that the cavefish represent a naturally "sleep deprived" population, the cavefish in this study are not sleep deprived, rather they are adapted to a condition of reduced sleep relative to fish from surface populations. Comparing the effects of depriving fish from each morph on markers of DNA damage and repair, on gut reactive oxygen species, and on gene expression will be necessary to solidify any proposed link of these phenotypes to sleep.

A second important aspect that limits the interpretability and impact of this study is the absence of information about circadian variations in the parameters measured. A relationship between circadian phase, light exposure and DNA damage/repair mechanisms is known to exist in *A. mexicanus* and other teleosts, and for differences to exist between the cave and surface morphs in these phenomena (Beale et al. 2013, doi: 10.1038/ncomms3769).

Although the present study mentions that their experiments do not align with these previous findings, they do not perform the appropriate experiments to determine if this such a misalignment is genuine. Specifically, Beale et al. 2013 showed that white light exposure drove enhanced expression of DNA repair genes (including cpdp which is prominent in the current study) in both surface fish and cavefish morphs, but that the magnitude of this change was less in the cave fish because they maintained an elevated expression of these genes in the dark, whereas darkness suppressed the expression of these genes in the surface fish. If such a phenomenon is present in the setting of the current study, this would likely be a significant confound for the UV-induced gene expression experiments in intact larvae, and undermine the interpretation of the results derived from these experiments: as samples are collected 90 minutes after the dark-light transition (ZT 1.5) it would be expected that both cavefish and surface fish larvae should have a clear induction of DNA repair genes (including cpdp) regardless of 90s of UV exposure. The data in supplementary figure 3 is not sufficient to discount this potentially serious confound, as for larvae there is only gene expression data for timepoints from ZT2 to ZT 14, with all of these timepoints being in the light phase and not capturing any dynamics that would occur at the most important timepoints from ZT0-ZT1.5, in the relevant period after dark-light transition. Indeed, an appropriate control for this experiment would involve frequent sampling at least across 48 hours to assess light-linked and developmentally-related changes in gene expression that would occur in 5-6dpf larvae of each morph independently of the exposure to UV.

On a broader point, given the effects of both circadian rhythm and lighting conditions that are thought to exist in *A. mexicanus* (e.g. Beale et al. 2013) experiments involving measurements of DNA damage and repair, gene expression, and reactive oxygen species etc. at multiple times across >1 24 hour cycle, in both light-dark and constant illumination conditions (e.g. constant dark) would be needed to substantiate the authors' interpretation that their findings indicate consistently altered levels of these parameters in the cave fish relative to the surface fish. Most of the data in this study is taken at only single timepoints.

In summary, the authors show that there are differences in gene expression, activity of DNA damage response and repair pathways, response to UV radiation, and gut reactive oxygen species between the Pachón cavefish morph and the surface morph of *Astyanax mexicanus*. However, the data presented does not make the precise nature of these differences very clear, and the interpretation of the results appears to be overly strong. Furthermore, the evidence of a link between these morph specific differences and sleep is unconvincing.

Comments on revisions:

I thank the authors for their engagement with the notes and recommendations I made in my original comments. I have no further recommendations to make here.

<https://doi.org/10.7554/eLife.99191.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

Abstract

I don't think you need the first two sentences of the abstract. This is not a grant and your results are exciting enough to justify a full basic science-based approach.

We fully understand this perspective. However, we prefer to introduce the work in the broader context of sleep medicine. This manuscript is part of our long-standing efforts to develop cavefish as a model for sleep disorders and we believe this provides important context.

Last sentence of the abstract: the subject is missing. "That have developed..." who has developed?

Thank you. We have corrected this error, the sentence now reads "...these findings suggest that cavefish have developed resilience to sleep loss..."

Introduction

First paragraph. Worth explaining in a sentence what is the link between DNA damage and ROS.

We now state 'Further, chronic sleep loss results in elevated reactive oxygen species (ROS), a known mediator of DNA damage, in the gut and/or brain that contribute to mortality in *Drosophila* and mice [11,16].'

"A. mexicanus exists as blind cave populations and an extant surface population that are interfertile". This needs rephrasing. As it is, it sounds like the surface population is infertile.

We have rephrased for clarity; the line now reads: "while the surface and cave populations are geographically isolated, they remain interfertile and capable of hybridization in nature as well as laboratory settings".

"Further, the evolved differences in DNA repair genes, including links between mechanisms regulating sleep, light responsiveness, and DNA repair across all three cave populations studied to date [27,29]" This sentence is incomplete.

We have corrected the phrasing, which now reads "...evolved differences in DNA repair genes have been identified across all three cave populations studied to date, including links between mechanisms regulating sleep, light responsiveness, and DNA repair":

Figure 1

I recommend improving the legibility of the figure copying some of the information provided in the legend directly within the figure itself.

A, B: label in the panel itself what is blue and what is green.

Thank you, we have made this change.

C: Make it clear in the figure itself that you are measuring yH2AX. Also, probably you have enough room in the figure to avoid abbreviations for Rhomb, mes, and tele. It may also help if you could add a little cartoon that explains what those three brain regions are.

We have added text to the y axis indicating that yH2AX fluorescence is being measured, and replaced the abbreviations with the full names of the regions.

G: again, explain that DHE is being measured here. And perhaps pick a different colour choice to highlight the difference from C?

We have added clarification to the y-axis of the figure, but have retained the color scheme for consistency; in all surface-cave comparisons in the manuscript, gray is used for surface fish and red for cavefish.

In the text: I would recommend adding some quantitative reminder of what is the difference in sleep amount between the two species (cave vs surface).

We have added the following to highlight the magnitude of the difference in sleep: “Strikingly, cavefish sleep as little as 1-2 hours per day, in contrast to their surface counterparts, which sleep as much as 6-10 hours a day”

"Together, these findings fortify the notion that cellular stress is elevated in the gut of cavefish relative to surface fish." Were the two populations fed the same diet and raised in the same lab conditions? If this is pinpointed to sleep amount, it's worth ruling out possible confounding factors.

We have added a sentence to the results underlining this point: “Prior to imaging, both surface and cavefish had been reared in a temperature-controlled incubator, and relied solely on their yolk sac for nutrients; so, differences in gut ROS cannot be attributed to differences in rearing or feeding conditions.”

Figure 2

Spell out, somewhere in the figure itself, that the 30s and 60s refer to UV treatment protocols.

We have added X-axis titles to clarify this in Fig 2 and supp. Fig 1.

It would be worth providing a cartoon of the experimental setup that shows for instance what time of the day UV was given (it's only specified in the text) and which subsequent sleep period was selected for comparisons.

We have added arrows to all sleep plots indicating the time of UV treatment, and brackets indicating the time period used for statistical comparisons, as well as text in the figure legends indicating this.

Figure 3

A. I don't think this is needed, to be honest, and if you want to keep it, it needs a better legend.

We have edited the figure legend to increase clarity.

B. I would make it clear in the figure that this refers to transcriptomics analysis. Perhaps you could change the order and show C, D, and then B.

We have added text to the figure legend and the results text to more explicitly state that the PCA plot is of transcriptional response. We have however retained the original figure order, as we feel this figure is important to establish that both populations have strong, but distinct responses to the UV treatment.

Figure 4

A. Spell it out in the figure itself that you're staining for CPD.

Thank you, we have made this change.

B. You are using the same colour combination you had in Figure 1 but for yet another pairing. This is a bit confusing.

Thank you for bringing this to our attention. We have added descriptions of the colors in the figure legend.

Discussion

"Beyond the Pachón cavefish population, all three other cavefish populations have been found to have reduced sleep (Cite)." Citation missing here.

Thank you. We have now clarified this sentence and included a citation.

Reviewer #2 (Recommendations For The Authors):

Consideration of Environmental Conditions:

Evaluate whether the lab conditions, which may more closely resemble surface environments, could influence the observed increase in neuronal DNA damage and gut ROS levels in cavefish. Adjusting these conditions or discussing their potential impact in the manuscript would strengthen the findings.

We are very excited about these experiments. We have a paper that will be submitted to *BioRxiv* this week where we record wild-caught fish, as well as fish in caves. The conclusion is that sleep loss is present in both populations. This field work took over 10 years to come together and still lacks the power of the lab based assays. Nevertheless, we can conclusively say that the phenotypes we have observed for the last ~15 years in the lab are present in a natural setting. We have included a statement about the need for future work to test these findings in a natural setting.

Alternative Stressors:

Given that cavefish are albino and blind (to my knowledge), consider using alternative sources of genotoxic stress beyond UV-induced damage. This could include chemical agents or other forms of environmental stress to provide a more comprehensive assessment of DDR.

We agree and are enthusiastic about looking more generally at stress. We note that we have previously found that cavefish rebound following sleep deprivation (McGaugh et al, 2020) suggesting that they are responsive to sleep disruption. This will be a major research focus area moving forward.

Broader Stress Responses:

Investigate whether other forms of stress, such as dietary changes or temperature fluctuations, elicit similar differences in sleep patterns and DDR responses. This could provide additional insights into the robustness of the observed phenomena.

We fully agree. This will be the primary focus of this research area moving forward. We hypothesize that cavefish are generally less responsive to their environment. Unpublished data reveals that temperature stress, circadian changes, and aging (presented here) to little to impact gene expression in surface fish. We would like to test the hypothesis that transcriptional stability of cavefish contributes to their longevity.

Potential Protective Mechanisms:

Discuss the possibility that lower levels of gamma-H2AX in cavefish might be protective, as DDR can lead to cellular senescence or cancer. This perspective could add depth to the interpretation of the results.

This was the hypothesis underlying this manuscript. However, we found elevated levels of gamma-H2AX. We believe there may be additional protective mechanisms that have evolved in cavefish, but cannot identify them to date. Our hope is future functional studies by our group, as well as other groups' access to this published work, may help address these questions.

Strengthening the Sleep-DNA Damage Link:

Further experiments are needed to directly link sleep differences to the observed variations in DNA damage and DDR. This could involve manipulating sleep patterns in surface fish and cavefish to observe corresponding changes in DNA repair mechanisms.

We agree. We have referenced work that conclusively showed this relationship in zebrafish. Our current methods for limiting sleep involves shaking, and this has too many confounds. We are working on developing genetic tools, and applying the gentle rocking methods used previously in zebrafish to address these questions.

Clarification of Causal Directionality:

Address the potential that sleep patterns and DDR responses may both be downstream effects of a common cause or independent adaptations to the cave environment. Clarifying this in the manuscript would provide a more nuanced understanding of the evolutionary adaptations.

Thank you for this suggestion. We have now added a paragraph describing how these experiments (and the ones described above) are necessary for understanding the relationship between sleep and DDR.

Clarification and Presentation:

Fix the many typos, and improve the clarity of the figures and their legends to ensure they are easily interpretable. Additional context in the discussion section would help readers understand the significance and potential implications of the findings.

Thank you, we have now included this.

Reviewer #3 (Recommendations For The Authors):

There are a number of suggestions that I have made in the public review, but there are a few things that I would like to add here.

The methods section is missing many important details, for instance, the intensity of the illumination used in the UV exposure in larvae is not reported but is vital for the

interpretation/replication of these experiments. In general, this section should be redone with a greater effort to include all important information. Similarly, the figure legends could be greatly improved, with important details like n-number and definition of significance thresholds defined (e.g. see Figures 1, C, and G.)

We have added greater detail to the methods section to specify the spectral peak and power output of the bulbs used.

There are a number of passages in the manuscript that do not make sense, which suggests that a future version of record should be carefully proofread. I know that this can be a case of reading multiple versions of a manuscript so many times that one doesn't really see it anymore, but, for example, phrases like "To differentiate between these two possibilities" are confusing to the reader when there has been no introduction of alternate possibilities.

Thank you for this comment. We have fixed this mistake and proofread the manuscript.

Additionally, there are multiple examples of errors in citations/references. A few examples are below:

"Further, chronic sleep loss results in elevated reactive oxygen species (ROS) in the gut and/or brain that contribute to mortality in Drosophila and mice [11, 16]". Reference 16 does not include mice at all, and reference 11 is Vaccaro et al. 2020, where Drosophila mortality is assessed, but mouse mortality is not.

We have added the appropriate citations and revised this sentence.

References 13 and 15 are the same.

Thank you, we have fixed.

References 24 and 26 are the same.

Thank you, we have fixed.

Public Reviews:

Reviewer #1 (Public Review):

Summary:

*Lloyd et al employ an evolutionary comparative approach to study how sleep deprivation affects DNA damage repair in *Astyanax mexicanus*, using the cave vs surface species evolution as a playground. The work shows, convincingly, that the cavefish population has evolved an impaired DNA damage response both following sleep deprivation or a classical paradigm of DNA damage (UV).*

Strengths:

The study employs a thorough multidisciplinary approach. The experiments are well conducted and generally well presented.

Weaknesses:

Having a second experimental mean to induce DNA damage would strengthen and generalise the findings.

Overall, the study represents a very important addition to the field. The model employed underlines once more the importance of using an evolutionary approach to study sleep and provides context and caveats to statements that perhaps were taken a bit too much for granted before. At the same time, the paper manages to have an extremely constructive approach, presenting the platform as a clear useful tool to explore the molecular aspects behind sleep and cellular damage in general. The discussion is fair, highlighting the strengths and weaknesses of the work and its implications.

We fully agree with this assessment. We are currently performing experiments to test the effects of additional DNA damaging agents. We hope to extend these studies beyond DNA-damage agents to look more generally at how animals respond to stress including ROS, sleep deprivation, and high temperature. This will be a major direction of the laboratory moving forward.

*The manuscript investigates the relationship between sleep, DNA damage, and aging in the Mexican cavefish (*Astyanax mexicanus*), a species that exhibits significant differences in sleep patterns between surface-dwelling and cave-dwelling populations. The authors aim to understand whether these evolved sleep differences influence the DNA damage response (DDR) and oxidative stress levels in the brain and gut of the fish.*

Summary of the Study:

The primary objective of the study is to determine if the reduced sleep observed in cave-dwelling populations is associated with increased DNA damage and altered DDR. The authors compared levels of DNA damage markers and oxidative stress in the brains and guts of surface and cavefish. They also analyzed the transcriptional response to UV-induced DNA damage and evaluated the DDR in embryonic fibroblast cell lines derived from both populations.

Strengths of the Study:

Comparative Approach:

*The study leverages the unique evolutionary divergence between surface and cave populations of *A. mexicanus* to explore fundamental biological questions about sleep and DNA repair.*

Multifaceted Methodology:

The authors employ a variety of methods, including immunohistochemistry, RNA sequencing, and in vitro cell line experiments, providing a comprehensive examination of DDR and oxidative stress.

Interesting Findings:

The study presents intriguing results showing elevated DNA damage markers in cavefish brains and increased oxidative stress in cavefish guts, alongside a reduced transcriptional response to UV-induced DNA damage.

Weaknesses of the Study:

Link to Sleep Physiology:

The evidence connecting the observed differences in DNA damage and DDR directly to sleep physiology is not convincingly established. While the study shows distinct DDR patterns, it does not robustly demonstrate that these are a direct result of sleep differences.

We agree with this assessment. We are currently working to apply tools developed in zebrafish to examine the physiology of sleep. While this is important, and our results are promising, we will note that functional analysis of sleep physiology in fish has been limited to zebrafish. We hope future studies will allow us to integrate approaches that examine the physiology of sleep.

Causal Directionality:

The study fails to establish a clear causal relationship between sleep and DNA damage. It is possible that both sleep patterns and DDR responses are downstream effects of a common cause or independent adaptations to the cave environment.

We agree, however, we note that this could be the case for all animals in which sleep has been linked to DNA damage. We believe the most likely explanation for *Astyanax* and other animals studied, is that sleep and DDR are downstream/interface with the sleep homeostat.

Environmental Considerations:

The lab conditions may not fully replicate the natural environments of the cavefish, potentially influencing the results. The impact of these conditions on the study's findings needs further consideration.

This is correct. We have considered this carefully. After nearly a decade of effort, we have completed analysis of sleep in the wild. These will be uploaded to BioRxiv within the next week.

Photoreactivity in Albino Fish:

The use of UV-induced DNA damage as a primary stressor may not be entirely appropriate for albino, blind cavefish. Alternative sources of genotoxic stress should be explored to validate the findings.

We have addressed this above. Future work will examine additional stressors. Both fish are transparent at 6dpf and so it is unlikely that albinism impacts the amount of UV that reaches the brain.

Assessment of the Study's Achievements:

The authors partially achieve their aims by demonstrating differences in DNA damage and DDR between surface and cavefish. However, the results do not conclusively support the claim that these differences are driven by or directly related to the evolved sleep patterns in cavefish. The study's primary claims are only partially supported by the data.

Impact and Utility:

The findings contribute valuable insights into the relationship between sleep and DNA repair mechanisms, highlighting potential areas of resilience to DNA damage in cavefish. While the direct link to sleep physiology remains unsubstantiated, the study's data and methods will be useful to researchers investigating evolutionary biology, stress resilience, and the molecular basis of sleep.

Reviewer #3 (Public Review):

*Lloyd, Xia, et al. utilised the existence of surface-dwelling and cave-dwelling morphs of *Astyanax mexicanus* to explore a proposed link between DNA damage, aging, and the*

evolution of sleep. Key to this exploration is the behavioural and physiological differences between cavefish and surface fish, with cavefish having been previously shown to have low levels of sleep behaviour, along with metabolic alterations (for example chronically elevated blood glucose levels) in comparison to fish from surface populations. Sleep deprivation, metabolic dysfunction, and DNA damage are thought to be linked and to contribute to aging processes. Given that cavefish seem to show no apparent health consequences of low sleep levels, the authors suggest that they have evolved resilience to sleep loss. Furthermore, as extended wake and loss of sleep are associated with increased rates of damage to DNA (mainly double-strand breaks) and sleep is linked to repair of damaged DNA, the authors propose that changes in DNA damage and repair might underlie the reduced need for sleep in the cavefish morphs relative to their surface-dwelling conspecifics.

To fulfill their aim of exploring links between DNA damage, aging, and the evolution of sleep, the authors employ methods that are largely appropriate, and comparison of cavefish and surface fish morphs from the same species certainly provides a lens by which cellular, physiological and behavioural adaptations can be interrogated. Fluorescence and immunofluorescence are used to measure gut reactive oxygen species and markers of DNA damage and repair processes in the different fish morphs, and measurements of gene expression and protein levels are appropriately used. However, although the sleep tracking and quantification employed are quite well established, issues with the experimental design relate to attempts to link induced DNA damage to sleep regulation (outlined below). Moreover, although the methods used are appropriate for the study of the questions at hand, there are issues with the interpretation of the data and with these results being over-interpreted as evidence to support the paper's conclusions.

This study shows that a marker of DNA repair molecular machinery that is recruited to DNA double-strand breaks (γ H2AX) is elevated in brain cells of the cavefish relative to the surface fish and that reactive oxygen species are higher in most areas of the digestive tract of the cavefish than in that of the surface fish. As sleep deprivation has been previously linked to increases in both these parameters in other organisms (both vertebrates and invertebrates), their elevation in the cavefish morph is taken to indicate that the cavefish show signs of the physiological effects of chronic sleep deprivation.

It has been suggested that induction of DNA damage can directly drive sleep behaviour, with a notable study describing both the induction of DNA damage and an increase in sleep/immobility in zebrafish (*Danio rerio*) larvae by exposure to UV radiation (Zada et al. 2021 doi:10.1016/j.molcel.2021.10.026). In the present study, an increase in sleep/immobility is induced in surface fish larvae by exposure to UV light, but there is no effect on behaviour in cavefish larvae. This finding is interpreted as representing a loss of a sleep-promoting response to DNA damage in the cavefish morph. However, induction of DNA damage is not measured in this experiment, so it is not certain if similar levels of DNA damage are induced in each group of intact larvae, nor how the amount of damage induced compares to the pre-existing levels of DNA damage in the cavefish versus the surface fish larvae. In both this study with *A. mexicanus* surface morphs and the previous experiments from Zada et al. in zebrafish, observed increases in immobility following UV radiation exposure are interpreted as following from UV-induced DNA damage. However, in interpreting these experiments it is important to note that the cavefish morphs are eyeless and blind. Intense UV radiation is aversive to fish, and it has previously been shown in zebrafish larvae that (at least some) behavioural responses to UV exposure depend on the presence of an intact retina and UV-sensitive cone photoreceptors (Guggiana-Nilo and Engert, 2016, doi:10.3389/fnbeh.2016.00160). It is premature to conclude that the lack of behavioural response to UV exposure in the cavefish is due to a

different response to DNA damage, as their lack of eyes will likely inhibit a response to the UV stimulus.

We believe that in *A. mexicanus*, like in zebrafish, it is highly unlikely that the effects of UV are mediated through visual processing. Even if this were the case, the timeframe of UV activation is very short compared to the time-scale of sleep measurements so this is unlikely to be a confound.

Indeed, were the equivalent zebrafish experiment from Zada et al. to be repeated with mutant larvae fish lacking the retinal basis for UV detection it might be found that in this case too, the effects of UV on behaviour are dependent on visual function. Such a finding should prompt a reappraisal of the interpretation that UV exposure's effects on fish sleep/locomotor behaviour are mediated by DNA damage.

We prefer not to comment on Zada et al, as that is a separate manuscript.

An additional note, relating to both Lloyd, Xia, et al., and Zada et al., is that though increases in immobility are induced following UV exposure, in neither study have assays of sensory responsiveness been performed during this period. As a decrease in sensory responsiveness is a key behavioural criterion for defining sleep, it is, therefore, unclear that this post-UV behaviour is genuinely increased sleep as opposed to a stress-linked suppression of locomotion due to the intensely aversive UV stimulus.

We understand this concern and are working on improved methodology for measuring sleep. However, behavioral measurements are the standard for almost every manuscript that has studied sleep in zebrafish, flies, and worms to date.

The effects of UV exposure, in terms of causing damage to DNA, inducing DNA damage response and repair mechanisms, and in causing broader changes in gene expression are assessed in both surface and cavefish larvae, as well as in cell lines derived from these different morphs. Differences in the suite of DNA damage response mechanisms that are upregulated are shown to exist between surface fish and cavefish larvae, though at least some of this difference is likely to be due to differences in gene expression that may exist even without UV exposure (this is discussed further below).

UV exposure induced DNA damage (as measured by levels of cyclobutene pyrimidine dimers) to a similar degree in cell lines derived from both surface fish and cave fish. However, γH2AX shows increased expression only in cells from the surface fish, suggesting induction of an increased DNA repair response in these surface morphs, corroborated by their cells' increased ability to repair damaged DNA constructs experimentally introduced to the cells in a subsequent experiment. This "host cell reactivation assay" is a very interesting assay for measuring DNA repair in cell lines, but the power of this approach might be enhanced by introducing these DNA constructs into larval neurons in vivo (perhaps by electroporation) and by tracking DNA repair in living animals. Indeed, in such a preparation, the relationship between DNA repair and sleep/wake state could be assayed.

Comparing gene expression in tissues from young (here 1 year) and older (here 7-8 years) fish from both cavefish and surface fish morphs, the authors found that there are significant differences in the transcriptional profiles in brain and gut between young and old surface fish, but that for cavefish being 1 year old versus being 7-8 years old did not have a major effect on transcriptional profile. The authors take this as suggesting that there is a reduced transcriptional change occurring during aging and that the transcriptome of the cavefish is resistant to age-linked changes. This seems to be only one of the equally plausible interpretations of the results; it could also be the case that

alterations in metabolic cellular and molecular mechanisms, and particularly in responses to DNA damage, in the cavefish mean that these fish adopt their "aged" transcriptome within the first year of life.

This is indeed true. However, one could also interpret this as a lack of aging. If the profile does not change over time, the difference seems largely semantic.

A major weakness of the study in its current form is the absence of sleep deprivation experiments to assay the effects of sleep loss on the cellular and molecular parameters in question. Without such experiments, the supposed link of sleep to the molecular, cellular, and "aging" phenotypes remains tenuous. Although the argument might be made that the cavefish represent a naturally "sleep-deprived" population, the cavefish in this study are not sleep-deprived, rather they are adapted to a condition of reduced sleep relative to fish from surface populations. Comparing the effects of depriving fish from each morph on markers of DNA damage and repair, gut reactive oxygen species, and gene expression will be necessary to solidify any proposed link of these phenotypes to sleep.

We agree this would be beneficial. We note that relatively few papers have sleep deprived fish. While we have done this before in *A. mexicanus* the assay is less than ideal and likely induces generalizable stress. We are working on adapting more recently developed methods in zebrafish.

*A second important aspect that limits the interpretability and impact of this study is the absence of information about circadian variations in the parameters measured. A relationship between circadian phase, light exposure, and DNA damage/repair mechanisms is known to exist in *A. mexicanus* and other teleosts, and differences exist between the cave and surface morphs in their phenomena (Beale et al. 2013, doi: 10.1038/ncomms3769). Although the present study mentions that their experiments do not align with these previous findings, they do not perform the appropriate experiments to determine if such a misalignment is genuine. Specifically, Beale et al. 2013 showed that white light exposure drove enhanced expression of DNA repair genes (including *cpdp* which is prominent in the current study) in both surface fish and cavefish morphs, but that the magnitude of this change was less in the cave fish because they maintained an elevated expression of these genes in the dark, whereas the darkness suppressed the expression of these genes in the surface fish. If such a phenomenon is present in the setting of the current study, this would likely be a significant confound for the UV-induced gene expression experiments in intact larvae, and undermine the interpretation of the results derived from these experiments: as samples are collected 90 minutes after the dark-light transition (ZT 1.5) it would be expected that both cavefish and surface fish larvae should have a clear induction of DNA repair genes (including *cpdp*) regardless of 90s of UV exposure. The data in Supplementary Figure 3 is not sufficient to discount this potentially serious confound, as for larvae there is only gene expression data for time points from ZT2 to ZT 14, with all of these time points being in the light phase and not capturing any dynamics that would occur at the most important timepoints from ZT0-ZT1.5, in the relevant period after dark-light transition. Indeed, an appropriate control for this experiment would involve frequent sampling at least across 48 hours to assess light-linked and developmentally-related changes in gene expression that would occur in 5-6dpf larvae of each morph independently of the exposure to UV.*

We agree that this would be useful, however, frequent sampling is not feasible given the experiments presented here and the challenges of working with an emerging model.

*On a broader point, given the effects of both circadian rhythm and lighting conditions that are thought to exist in *A. mexicanus* (e.g. Beale et al. 2013) experiments involving*

measurements of DNA damage and repair, gene expression, and reactive oxygen species, etc. at multiple times across >1 24 hour cycle, in both light-dark and constant illumination conditions (e.g. constant dark) would be needed to substantiate the authors' interpretation that their findings indicate consistently altered levels of these parameters in the cavefish relative to the surface fish. Most of the data in this study is taken at only single time points.

Again, see comment above. The goal was to identify whether there are differences in DNA Damage response between *A. mexicanus*. Extending on this to examine interactions with the circadian system could be a useful path to pursue in the future.

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*In summary, the authors show that there are differences in gene expression, activity of DNA damage response and repair pathways, response to UV radiation, and gut reactive oxygen species between the Pachón cavefish morph and the surface morph of *Astyanax mexicanus*. However, the data presented does not make the precise nature of these differences very clear, and the interpretation of the results appears to be overly strong. Furthermore, the evidence of a link between these morph-specific differences and sleep is unconvincing.*

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