nature portfolio

Peer Review File

DNA damage modulates sleep drive in basal cnidarians with divergent chronotypes

Corresponding Author: Professor Lior Appelbaum

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
What a treat! This was an enjoyable paper to read. The English would benefit from a more thorough proof-read as it was chunky at times, still the data are important and original. Here, the authors characterize sleep in upside-down jellyfish (done before by Nath et al 2017), but now also an anemone for which sleep data was indirect. They then look for the adaptive value of sleep, with focus on DNA repair. To do so they measured a DNA damage-proxy under baseline and sleep-deprivation conditions, but then flip-the-table to look at exogenous stimulation (UV light) as a trigger for sleep. Very nice. Here are some comments I hope will strengthen their solid paper.

Line 13: “of sleep to the simple nerve net”

Line 14: Please give common names before the scientific name; e.g., upside-down jellyfish and starlet sea anemone.

Line 16-17. The clock is not the dominant regulator of sleep in the anemone as you say on line 135.

Line 26. The earliest known jellyfish comes from the Cambrian, not Precambrian Period; see Moon et al., 2023 Proceedings of the Royal Society B. But in any case, Precambrian is no longer an accepted term for geological epochs. This term was replaced, in 2004, by the Ediacaran Period.

Line 28. Whether sleep loss is lethal is open to debate. The rats used in the three-weeks chronic sleep deoprivation study were confounded by periodic thermoregulatory challenged from repeatedly getting wet whenever they fell asleep.

Line 37: the presence of sleep homeostasis in nocturnal and diurnal animals does not mean there is a common evolutionary origin. Rather the presence of sleep and circadian regulation of sleep in basal and derived animals will suggest that…

Line 49: “symbiotic” implies that the jellyfish is the symbiont, whereas it is the photosynthetic dinoflagellates that are the symbionts of the jellyfish. Similarly, it should just be stated that the anemone studied does not contain symbiotic eukaryotes.

Line 54: It is said that the authors meticulously define sleep in the jellyfish, and yet later we find that sleep is defined simply as fewer than 37 pulses per minute over 3-minutes. I would argue that this is not specific at all: is sleep occurring between the pulses or also during a slow pulse? Given that sleep is a prominent behaviour characterized by restfulness (first and foremost) it is odd to say that jellyfish are sleeping when they still move, just less. I accept the results of the sleep homeostasis study in which stimulated jellyfish pulse less when allowed to behave freely, but it would be important to know what aspect of this recovery is the actual sleep: all of it, including slow pulses? Or a subset and if so, how much?

Line 71 and throughout the Results: Please give variance when giving means in the text.

Line 78-79: The meaning of “average sleep” is unclear: “Applying this criterion revealed that the average sleep increased during the night…”

Line 89-100: Looking at animals in the wild is a very nice component to see whether pulses in the lab are meaningful for the species in nature. I do wish you also applied the treatment in the wild too. Why not? Just bring the lights to the animals :)

Line 107-108: This sentence is clunky and could even be removed without loss of content.

(Line 111. In the future, you should simulate dawn and dusk in the lab with transitions between light and dark.)

Line 121: You have characterized sleep, not defined it. Use of the word ‘define’ is circular.

Line 124: To look at circadian regulation, normally, one would do many photoperiodic regimes, including 12:12 LD, 24:0 LL, 0:24 DD but also 6:6:6:6 (LDLD) and others. The latter is called forced desynchrony.

Line 144. Please remove the term ‘sleep quality’ throughout. This term originates in humans with the assumption that sleep continuity is the most important feature of sleep. In non-human animals, we have no idea what constitutes “high-quality” sleep or not. For instance, the recent report of 10,000 sleep bouts per day in penguins… is that high quality sleep or not? Ultimately, we have no idea, since the term is vague and best avoided. You could just say you measured the duration of sleep episodes.

Line 154. Melatonin is only sleep promoting in diturnal species, thus it is not surprising it had little effect in crepuscular aneneomes. It is important to note also (line 158) that melatonin has already been shown to increase sleep in jellyfish (Nath eta l 2017). That said, you could say nocturnal flatworms given melatonin show reduced activity (Omond et al 2017).

Line 233. I think the work Symbiodiniaceae can go since you refer to them first as dinoflagelattes.

Line 281-283. I think these last lies would benefit from expansion, even while speculative. All life is made of DNA and much of that life is transparent, microscopic, sea-living and exposed to sunlight rich in UV and X-rays. I’d like to hear more on the authors thoughts on where they think sleep began.

That's it! Thanks,
John

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
This paper represents the third reported instance of sleep phenomena in cnidarians, might be contributing significantly to the clarification of the evolutionary discourse on sleep. I assume the identification of sleep phenomena in both C. andromeda and N. vectensis likely demanded considerable effort. Moreover, beyond the mere reporting of sleep phenomena, the paper delves into the significance of sleep, noting the observed relationship between DNA damage and sleep in cnidarians, echoing findings in other organisms and addressing the early significance of sleep in animals.

However, given the significance of the research and its findings, meticulous attention to the paper's structure, methodology, analysis, statistics, and figure presentation is warranted. Particularly, the presence of notation in the statistics section that may not be readily understandable is regrettable. It is imperative that the presentation be crafted to allow readers to grasp the content instantaneously and clearly. Additionally, some roughness is evident in the experimental aspect concerning the relationship between DNA damage and sleep, prompting cautious consideration of the paper's claims. Below, I have enumerated specific concerns that stand out, suggesting that addressing these issues is essential at the very least.

Major:

1. The vertical axis of Fig.1c does not start from zero, giving the impression of a clear LD cycle, but in reality, the difference in pulses per minute is very small. The increase in activity immediately after the light period and before the dark period suggests that much of the difference between day and night in Fig1.d is due to this rise in activity. This bimodal activity, also observed in organisms like Drosophila, often signifies time recognition by the circadian clock. However, as the authors themselves note, based on the results in Extended Data Fig. 1a-d, it seems unlikely that a circadian rhythm is controlling behavior in C. andromeda. In that case, how should we interpret the bimodal pattern seen in Fig1.c? Given its likely significant impact on the difference between day and night in Fig1.d, it should be adequately explained. For instance, it would be advisable to investigate what activity and sleep cycles are observed when the LD cycle is changed to 6h:6h or 3h:3h instead of 12h:12h.

2. In Figures 1o and p, it is not easy to find explanations regarding sample size and statistics, making it difficult to properly evaluate the results. To assert the validity of the results, descriptions regarding sample size and statistics should be made explicit.

3. The mentioned statement, particularly "36.4 pulses/min, Fig. 1q", refers to a specific data point in Figure 1q where the average pulse rate during the day at sea is represented. This data point is crucial for understanding the comparison being made in the sentence. Additionally, it is indeed conceivable that locomotor activity (measured in pulses/min) might be influenced not only by bell size but also by temperature variations. Therefore, it would be prudent to discuss the potential

impact of seawater temperature (changes) on locomotor activity.

4. In the experiments presented in Fig.2f-k, the latency to response when stimuli were applied to individuals in an active state cannot be considered as a response time. Therefore, the latency to response when the Length of quiescent bout is 0 should be excluded. Its inclusion would naturally result in shorter response times when the quiescent bout is less than 8 minutes, significantly affecting the definition of sleep. Especially considering that Fig.3 also measures bout length, there is concern that this inclusion may influence other results as well.

5. In Fig2.l-q, if discussing the sleep patterns of DD and NvClk mutant, it would be beneficial to include their activity rhythms as well. This would enhance clarity and facilitate better understanding of the data.

6. In the experiments presented in Fig.2r, and s, how were the close tentacle and the open tentacle distinguished from the imaging data? Is it possible that the difference in sleep quantity between them is due to the detection of tentacle movement as activity, leading to an underestimation of sleep?

7. The statistics regarding the results in Fig.2t, and u are not readily apparent, making it difficult to evaluate the results properly. While it is possible that they are mentioned somewhere, it is not immediately clear, so please ensure that they are presented clearly for easy understanding.

8. In Fig.3c, e, and g, why are mean transitions presented as count per hour? In this case, it seems that representing the number of bouts per 12 hours would be the simplest and most basic way of notation.

9. In Fig3j, kl, and m, as melatonin is known to influence circadian rhythms, it is important to show the sleep patterns when melatonin is added, not only to determine its effect on sleep but also to clearly distinguish its influence from that of circadian rhythms. Additionally, I feel a slight discomfort with the fact that detailed analyses such as day versus night or ZT6-ZT18 versus ZT18-ZT6 have not been conducted, which would provide a comparison with previous analyses.

10. The figures in Fig. 4a and b are difficult to understand immediately, so please make efforts to create figures that are easier for readers to comprehend. It is unclear why, despite both graphs having the same vertical axis labeled as 'average sleep time during 12h preceding each time point,' the range of transitions in (a) is approximately 40% to 60%, while in (b) it remains between 15% and 30%. Additionally, the statistics regarding the results in Fig.4g and h are not readily apparent, making it difficult to evaluate the results properly. While they may be mentioned somewhere, it is not immediately clear, so please ensure that they are presented clearly for easy understanding.

11. The figures in Fig. 4g and h exhibit a considerable number of outliers, giving the impression that the discussion is based on very marginal differences. Therefore, I believe there might be room for improvement in the analysis method or experimental conditions. If the authors' claims regarding the experimental results in Fig. 4 are accurate, providing continuous sleep deprivation (SD) could potentially lead to a higher accumulation of DNA damage. Conducting long-term SD experiments might enhance the credibility by demonstrating clearer differences.

12. It is difficult to determine whether DNA damage is involved in sleep based solely on the experimental method in Fig. 5. Changes in locomotor activity due to UV irradiation should also be included. Additionally, observing the relationship between the number of γH2AX foci on the day after UV irradiation and two days after UV irradiation and sleep would provide a more accurate discussion of the relationship between DNA damage and sleep.

Minor:
1. In LL and DD conditions, periodic activity is not observed in C. andromeda (Extended Data Fig. 1a-d). Does this suggest that clock genes are not present in the genome? Additionally, in LL conditions, the pulse/min is extremely high at the initial LD stage. Moreover, the sample size is n=6, smaller compared to others, raising some doubts about the reliability of these resultsPlease explain what the black triangles in Fig. 1e represent, as it is unclear.
2. Since the term 'optimization algorithm' may not be easily understood by general readers, could you provide a more specific description for clarity?
3. In LL and DD conditions, periodic activity is not observed in C. andromeda (Extended Data Fig. 1a-d). Does this suggest that clock genes are not present in the genome? Additionally, in LL conditions, the pulse/min is extremely high at the initial LD stage. Moreover, the sample size is n=6, smaller compared to others, raising some doubts about the reliability of these results.
4. In Extended Data Fig. 1c, it should be described as 'Circadian time (h)' under constant conditions. Additionally, Extended Data Fig. 1f should be described as 'time of day (h)' instead of 'Zeitgeber time (h).'
5. I do not understand the meaning of the red dashed lines in Extended Data Fig. 2f-k. It is necessary to describe them more clearly for better understanding.
6. L131: The text states 'ZT0-ZT8,' indicating an 8-hour sleep deprivation period during the day, while the legend mentions 'ZT0-ZT7.' To avoid confusion, it should be clarified which one is correct.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
This manuscript examines the link between DNA damage in neurons and sleep, extending previous observations that were made in zebrafish and rodent models to more “ancient” sleepers, the jellyfish and anemone. Overall, the work is intriguing and is suggestive of a link between sleep and DNA damage even in these animals that lack brains. In particular, the work demonstrating a sleep like state in the anemone is well done (with a few minor gaps, see minor comments) and the jellyfish behavioral work is a nice confirmation and extension of Nath et al., 2017. However, although problems with the data presentation and analysis of the DNA damage data makes it a little difficult to evaluate fully, the results don’t seem to line up with the major hypothesis that DNA damage is resolved during sleep in these species, and in fact some of the data seems to contradict that notion entirely.

The first half of the manuscript, which describes the sleep state in jellyfish and the anemone is done to a high standard, and all the major expected behavioral criteria are examined (e.g. circadian period, sleep deprivation and recovery, arousal threshold changes). This makes for a convincing case that the two species have sleep states, although one is diurnal and the other is more crepuscular. I have only minor concerns about some of the sleep deprivation methodology (see Additional comments below) and a bit more concern about the interpretation of the role of melatonin in the two species. They conclude that melatonin is effective as a hypnotic in the jellyfish (which was also shown by Nath et al., 2017) but not in the anemone; however, the timing of the melatonin delivery may have missed the effective window—adding melatonin at ZT2-5 puts this at the sleep trough for jellyfish but at a relatively high sleep window for the anemone. In other words, sleep could be ceilinged out so melatonin can’t boost sleep further, giving an artificial null result (adding melatonin to diurnal species like zebrafish also only really boosts sleep during the day and not at night when sleep and natural melatonin are already high). Testing melatonin later in the day, when sleep is lower, should be a straightforward way to bolster this claim.

The second half of the manuscript then examines the link between DNA damage and sleep, and here I find the analysis and interpretation of the results to be more muddled. Firstly, the independent experimental Ns for calculating DNA damage appears to be each cell (Figure 4g,h), but in how many independent animals? It isn’t really appropriate to treat cells from the same animal as independent experimental replicates for statistical purposes; a more appropriate analysis would be average foci/cell calculated for each animal, and the N would be each animal. Thus, it is difficult from the data as presented to conclude if there are true differences in some of the conditions following sleep deprivation. However, even if we take these results as described, some of the data appears to contradict the claim that sleep is important for resolving the DNA damage. For example, Figure 2t shows that a sleep deprivation from ZT0-6 leads to a substantial sleep rebound from ZT12-24. And yet, In figure 4h, the number of DNA damaged foci remains elevated at ZT17 following SD, even though these animals slept a lot more than the controls. This seems to contradict the conclusion of lines 180-183: “These experiments show that DNA damage increased during wakefulness and decreased during sleep in either diurnal or crepuscular cnidarians. Furthermore, these findings suggest that alleviating the levels of DNA damage, which accumulated during wakefulness, is an evolutionarily conserved cellular role of sleep”

Finally, the authors use UV light to induce DNA damage and claim that sleep is increased, thus showing a bi-directional relationship between DNA damage and sleep. However, the data are shown only as box-whisker summary plots, making them difficult to evaluate in terms of quality of the results. Better would be to display these as timecourse data, before and after UV exposure, and for a longer window than the summary—the post UV sleep is reaching 100% for jellyfish and 60/60 minutes for the anemone, so more convincing evidence that these animals are not permanently harmed by the UV exposure would be critical here. Even better would be to show that the DNA damage eventually resolves (by the next day or something) and that the sleep recovers as well.

To be more convincing, it would also be good to show that DNA damage induced in another manner also increases sleep—for example these creatures have a host of natural behavioral responses to UV light (see for example Lilly et al., 2024, where the larval anemones shift their behavior to a planktonic swimming in direct response to UV), so showing the sleep changes are due to the damage and not UV-activated neural pathways would go far to convince.

To conclude, I am convinced that anemones (and jellyfish) have a sleep state, and this alone is a nice contribution. However, I am less convinced that the DNA-damage sleep hypothesis has been so clearly demonstrated in these animals based on the current data presented.

Additional and minor comments:

Figure 2—The arousal threshold data, circadian and clock mutant data are all very good. The Sleep deprivation data is good but a few additional controls would be useful. For example, the rebound is shown after depriving the animals of the daytime siesta, but the sleep traces show a lot of sleep in the latter half of the night as well—even more in fact. Showing SD for this window would also be good, as in other species like Drosophila is not totally clear that the siesta and night sleep are identically regulated. Perhaps more important is to use the SD method at a time of relative wakefulness and control for rebound due to physical exhaustion of being kept awake, a standard test in the field.

Figure 2—the SD method uses light, which may be altering circadian clock function and timing as well as sleep deprivation. Either a non-light method for SD should be used, or show that clocks aren’t shifted by the light pulses.

Figure 2- methods; the details for the light stimulation SD are not provided in the methods as far as I can see, including light

levels, etc.

Figure 2t—if light is being used to sleep deprive, it isn't clear to me why there is a gap in the data—it would be better to track the animals during the SD to see exactly how much sleep is lost. I can understand why there is a gap in Figure 1, where the SD is done physically that will interfere with the tracking, but light should be straightforward to track.

Figure 2m—defining the windows of analysis across the dark-light transition (ZT18-6 and 6-18) is a bit odd, I would just break it down into night 2nd half and day first half to be a little clearer.

Figure 3J—100 mM melatonin must be a typo, and it is listed as 100uM in the methods (and that could still be considered high). To convince that melatonin plays no role in the sleep of the anemone, adding melatonin at different timepoints will be critical (see main comments).

The term "sleep quality" is too loaded a term—it implies something about a "good" night or a "bad" night of sleep but it is impossible to say that fragmented sleep or consolidated sleep is of more or lesser quality without showing effects on performance the next day. I would recommend "sleep structure" or "sleep architecture" or similar when looking at sleep bout counts and lengths.

Figure 4: The SD experiment for jellyfish is from ZT17-23, but for N vectensis is ZT0-8. ZT0-8 does not quite line up with maximal sleep in this species ZT18-24 or ZT18-6 makes better alignment. Figure 4A, showing the rolling 12 hour pervious sleep time is misleading on this – the figure 4b data is high already at ZT0 and peaking at ZT6, but that means the sleep is maximal in the PREVIOUS 12 hours, which is when the SD should be done for testing the effect of SD on sleep. Plotting it this way is visually confusing compared to Figure 2L, where the sleep timing is easier to appreciate.

Figure 4: If sleep is really important for repairing the DNA damage, then why does it not recover in the anaemone after SD from ZT0-8? It is confusing because they sleep more from 12-17 then they even do during the "SD" ZT0-8 window, so if this were all about sleep driving the recovery, then wouldn't we expect it to be recovered? Figure 3 shows after SD, the anemones sleep more, so this should have resolved the foci if the interpretation were correct. This seems counter to the conclusion of line 180-183: "These experiments show that DNA damage increased during wakefulness and decreased during sleep in either diurnal or crepuscular cnidarians. Furthermore, these findings suggest that alleviating the levels of DNA damage, which accumulated during wakefulness, is an evolutionarily conserved cellular role of sleep.

Figure 4: The data is not showing independent Ns…. The legend says n=291 cells, but how many animals? One cannot use a cell in the same animal as an independent measure for the stats unless you control for them as dependent on each other. What stats are being done in Figure 4? The legend doesn't say. It says Tukey, but that is a post-hoc test. It also lists a lot of cross comparisons, how is this being controlled for multiple comparisons? There are really two-factors, SD/no SD and time, so a 2-factor analysis design would be clearer.

Figure 5: the post-UV sleep data is too summarized. IT would be better to see the time course of the behavior afterwards. Also, the recovery on the following day—sleep is being driven so high after UV are you sure they aren't permanently harmed, i.e. sleeping a lot but actually damaged?

5e and 5f are not presented in the same manner, one show ZT8-12 and ZT12-0, the other says Pre: Post, but over what windows constitute "pre" and what consistutes "post"? The legend says 1hr pre and 1 hr post, but that seems like too short a time? What would be better would be to induce UV damage, see how long the damage takes to be resolved and correlate that timecourse with the sleep timecourses— i.e. once the damage is resolved, does sleep end?

Another method to induce DNA damage? These species are quite sensitive behaviorally to UV light directly, for example the anemone in the larval state are induced by UV into plaktonic swimming (Lilly et al 2024). Can you distinguish between these two models UV DNA damage sleep vs. UV sleep (independent of the DNA damage?

The evolutionary claim seems too strong: "Altogether, these inter-species comparative findings suggest that environmental stressors, such as UV radiation, which can induce DNA damage and cellular stress, drove the evolutionary origin of sleep in the day or crepuscular active cnidarians." What exactly is the evidence that this was the "driver" of evolution? Showing that DNA damage induces sleep and sleep resolves DNA damage in these species isn't quite the same as saying that this relationship is what "drives" evolution of sleep.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have effectively addressed my concerns. I have nothing new to add.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The revised manuscript has addressed many of the concerns raised in the previous round, and I acknowledge a clear overall improvement in both clarity and scientific rigor. In particular, the experimental validation of sleep phenomena in C. andromeda and N. vectensis appears robust and convincing. The study adds valuable insights into sleep regulation in cnidarians and constitutes a meaningful contribution to the field.

However, regarding the central claim expressed in the title—namely, that "DNA damage is a sleep driver in ancient cnidarians"—I believe further caution and refinement are required. Specifically, this claim demands greater precision and strength in both experimental design and data analysis. I outline below several key issues that I recommend the authors address in the current revision.

1. Y-axis scaling and quantification in Figure 4i,j
The vertical axis in Figure 4i,j lacks sufficient explanation, making it difficult to interpret. If the same quantification method (i.e., puncta/cell) as in Figure 5d–f was used, then I suggest that the graphical representation should be standardized across figures for consistency.
Conversely, if a different quantification method was employed, then the authors should explain why the approach used in Figure 5 was not applied here. In that case, the rationale for using an alternative method should be clearly justified from a scientific standpoint. Overall, the quantification approach should be unified where possible.

2. Statistical annotation (Figure 4i,j and other figures)
The notation "different lowercase letters above the bars indicate statistical significance at $p < 0.05$, Tukey HSD, two-tailed; FWER controlled" is vague and difficult to interpret. I strongly recommend that the authors explicitly state which groups were compared and the nature of the statistically significant differences.
Similar statistical annotations appear in other figures, including supplementary figures. A more intuitive and consistent presentation would enhance interpretability across the manuscript.

3. Relationship between sleep pressure and DNA damage
The current manuscript presents compelling evidence that DNA damage accumulates during wakefulness and is reduced during sleep under light–dark conditions. It also demonstrates that short-term sleep deprivation (SD) induces DNA damage, supporting the hypothesis that sleep contributes to DNA repair. These are important findings.

However, to convincingly support the central claim that "DNA damage is a sleep driver," I believe further experimentation is needed. Specifically, I encourage the authors to explore whether prolonged sleep deprivation—e.g., over 24 hours or multiple days—leads to a measurable accumulation of DNA damage. This would provide critical support for the proposed causal relationship between sleep pressure and DNA damage.

Furthermore, the authors have shown that melatonin induces sleep during the active phase but has no effect on sleep duration during the sleep phase. This feature could be leveraged experimentally. For example:

Does melatonin-induced sleep during the active phase reduce DNA damage?

Does melatonin administration during the sleep phase alter DNA damage independently of sleep induction?

These complementary experiments would allow the authors to distinguish between the antioxidative effects of melatonin and the role of sleep per se in promoting genomic stability. I believe that these additions would significantly strengthen the manuscript and make the central claim more persuasive.

4. Number of animals used in DNA damage assays
While the number of cells analyzed appears sufficient, the number of animals examined seems relatively low. Given that sleep-related behavioral metrics (e.g., bout duration, total sleep time) were calculated from a large number of animals, the DNA damage experiments should ideally match that level of biological replication.
To ensure comparability and statistical robustness, I recommend increasing the number of animals used in the DNA damage assays to align with those used in the sleep behavior analyses.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors have mostly addressed the reviewers' comments and my concerns, including with important new experiments that greatly improve the manuscript. With one very important exception that must be resolved before I could consider acceptance of this paper.

In my original review, I raised an issue of statistical pseudoreplication that comes from treating neurons within the same animal as “independent”. Their response was not to correct this but instead claim that they “focus on a cellular phenotype” and that “each neuron independently accumulates and repairs DNA damage and is therefore an individual experimental unit.” I very strongly disagree with this claim, on scientific first principles. In support, the authors state this is an established method by citing their own papers! Even if this is something that was done in other papers, it doesn’t make the use of this approach in this paper right; it just means that those other papers are also doing this analysis incorrectly.

A few simple thought experiments can illustrate the point that neurons in the same animal are not, and cannot, be treated as statistically independent. First, consider an experiment where you expose some hydra to UV to see if DNA damage is resolved by sleep. At the first timepoint post UV, you grab a single animal that happened to be closer to the UV source for the duration of the experiment, and then you count DNA damage foci in 100s of neurons in that single animal. Now, on the second timepoint after sleep, you grab a single hydra that happened to stay far from the UV source (darn chance!), and count 100s of neurons. Is the n=100s for each group, or is the n=1? If you use n=100, even a very tiny effect, possibly caused by differences in UV exposure alone, will have some very small p-value, and you will rejoice that your experiment “worked”. But if you use n=1, you will correctly surmise that the n is too small to draw a meaningful conclusion and you won’t be misled by a chance result.

Even though the idea that n=100 can come from one animal already makes the point pretty obvious, maybe one can try to argue that all the animals must get the same UV. Consider a second thought experiment then, in which a single neuron in an animal gets zapped by UV that generates some DNA damage that causes it to become hyperactive. This single neuron then causes downstream neurons to become active as well, which we know can trigger DNA damage in those cells. You see? The DNA damage accumulating in neurons doesn’t happen independently of the other neurons in that animal, so they cannot be treated as independent. To do so inflates artificially the n, which for statistical purposes must be independent of each other. This is why in Figure 4 and 5, the authors report such small p-values, despite a fairly variable set of data—the n is inflated.

This fundamental misunderstanding does make me worried about how other analyses were done in the paper, but I don’t see anything clearly wrong in other figures.

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have responded thoughtfully and comprehensively to the points I raised in my reviews. Their additional experiments and revisions have successfully reinforced the manuscript's main conclusions. Furthermore, the revised title now better represents the content and focus of the study.

I have no additional comments or concerns. I support publication of this manuscript as currently revised.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors have addressed all of my concerns.

(Remarks on code availability)

Point-by-point response to the reviewer comments

Reviewer #1 (Remarks to the Author):

What a treat! This was an enjoyable paper to read. The English would benefit from a more thorough proof-read as it was chunky at times, still the data are important and original. Here, the authors characterize sleep in upside-down jellyfish (done before by Nath et al 2017), but now also an anemone for which sleep data was indirect. They then look for the adaptive value of sleep, with focus on DNA repair. To do so they measured a DNA damage-proxy under baseline and sleep-deprivation conditions, but then flip-the-table to look at exogenous stimulation (UV light) as a trigger for sleep. Very nice. Here are some comments I hope will strengthen their solid paper.

Response:

We thank the reviewer for their kind words and strong support. As requested, we have performed additional English language editing.

Comment:
Line 13: “of sleep to the simple nerve net”

Response:
The typo has been corrected in Page 1, Line 12.

” Considering the survival risks, why sleep evolved in basal lineages and what essential benefits it provides to the simple nerve net of nocturnal and diurnal invertebrates remain elusive”.

Comment:
Line 14: Please give common names before the scientific name; e.g., upside-down jellyfish and starlet sea anemone.

Response:
The common names have been added to the abstract, Page 1, Line 14.

“We used behavioral criteria to empirically define sleep in the upside-down jellyfish Cassiopea andromeda and the starlet sea anemone Nematostella vectensis.”

Comment:
Line 16-17. The clock is not the dominant regulator of sleep in the anemone as you say on line 135.

Response:
This sentence has been revised in Abstract, Page 1, Line 17.

“In contrast, both the circadian clock and homeostatic processes regulated sleep in N. vectensis, which increased sleep at dawn.”

Comment:
Line 26. The earliest known jellyfish comes from the Cambrian, not Precambrian Period; see Moon et al., 2023 Proceedings of the Royal Society B. But in any case, Precambrian is no longer an accepted term for geological epochs. This term was replaced, in 2004, by the Ediacaran Period.

Response:
As noted by the reviewer, the term referring to geological epochs has been replaced, and the relevant citation has been added in Page 1, Line 28.

*"...suggesting that sleep is a **Cambrian** metazoan adaptation[1–6]."*

Comment:
Line 28. Whether sleep loss is lethal is open to debate. The rats used in the three-weeks chronic sleep deprivation study were confounded by periodic thermoregulatory challenged from repeatedly getting wet whenever they fell asleep.

Response:
The description of the effect of sleep loss on health has been revised in Page 1, Line 29.

"Sleep deprivation (SD) and disturbances are associated with increased risk for health decline in bilaterian animals".

Comment:
Line 37: the presence of sleep homeostasis in nocturnal and diurnal animals does not mean there is a common evolutionary origin. Rather the presence of sleep and circadian regulation of sleep in basal and derived animals will suggest that…

Response:
This sentence has been revised in Page 2, Line 38.

"The presence of sleep and these regulatory processes in both basal and derived nocturnal and diurnal species suggests a common evolutionary origin of sleep regulation[16,17]"

Comment:
Line 49: "symbiotic" implies that the jellyfish is the symbiont, whereas it is the photosynthetic dinoflagellates that are the symbionts of the jellyfish. Similarly, it should just be stated that the anemone studied does not contain symbiotic eukaryotes.

Response:
This content has been added to the Introduction in Page 2, Line 53.

"The jellyfish Cassiopea, which lives in obligates symbiosis with photosynthetic dinoflagellates, exhibits rhythmic daily activity that peaks during the day. In contrast, the sea anemone Nematostella vectensis (N. vectensis), which lacks symbiotic eukaryotes, is considered nocturnal".

Comment:
Line 54: It is said that the authors meticulously define sleep in the jellyfish, and yet later we find that sleep is defined simply as fewer than 37 pulses per minute over 3-minutes. I would argue that this is not specific at all: is sleep occurring between the pulses or also during a slow pulse? Given that sleep is a prominent behaviour characterized by restfulness (first and foremost) it is odd to say that jellyfish are sleeping when they still move, just less. I accept the results of the sleep homeostasis study in which stimulated jellyfish pulse less when allowed to behave freely, but it would be important to know what aspect of this recovery is the actual sleep: all of it, including slow pulses? Or a subset and if so, how much?

Response:
Sleep in mobile animals is well-documented in migrating birds and marine mammals, which exhibit unihemispheric sleep[1]. Even humans move during sleep. Our raw data show that Cassiopea rarely pauses for more than one minute; nevertheless, it consistently reduces activity during sleep. By evaluating all behavioral sleep criteria, including an increased arousal threshold, we defined sleep in Cassiopea as fewer than 37 pulses per minute over a three-minute period. This threshold captures a state of reduced activity and aligns with the recovery behavior observed following sleep deprivation. We consider sleep to encompass the entire recovery period below this threshold, including both behavioral pulses and pauses. This is consistent with sleep definitions in basal animals, where molecular and physiological markers are often limited. We agree with the reviewer that characterizing potential sleep stages (pulses vs. pauses?) in Cassiopea is a promising research direction. We believe that this study lays essential groundwork for the development of molecular and physiological tools to define sleep architecture at both the cellular and behavioral levels in cnidarians.

To avoid the expectation that we would define sleep stages, we have removed the word "meticulously", Page 2, Line 59. The implications for future research on sleep architecture are now discussed in the Discussion section in Page 10, Line 337.

Comment:
Line 71 and throughout the Results: please give variance when giving means in the text.

Response:
We agree that reporting a measure of dispersion with every mean improves clarity. Accordingly, we have added "mean ± SEM (n = …)" after each numerical mean in the Results section and, where appropriate, in the figure legends. For example, in the results section page 03, line 77: *"locomotor activity consistently decreased at night, with a mean pulsation rate of 36.4 ± 1.6 pulses min^{-1} (mean ± SEM, n = 30) during the day and 31.9 ± 1.4 pulses min^{-1} (n = 30) at night (Fig. 1c,d)."*

Comment:
Line 78-79: The meaning of “average sleep” is unclear: “Applying this criterion revealed that the average sleep increased during the night…”

Response:
We intended to refer to sleep time. The sentence has been revised to: “*Applying this criterion revealed that the average sleep* ***time*** *increased during the night*”. Page 03, Line 86.

Comment:
Line 89-100: Looking at animals in the wild is a very nice component to see whether pulses in the lab are meaningful for the species in nature. I do wish you also applied the treatment in the wild too. Why not? Just bring the lights to the animals :)

Response:

Cassiopea lives in open seawater, and conducting arousal threshold experiments requires video recordings under controlled conditions to ensure consistent measurements. Performing such experiments in the wild is technically challenging due to environmental variables such as the presence of other animals, light fluctuations, sediment, and water turbidity. Additionally, locating and maintaining the animal within the experimental area is non-trivial. While technically feasible, implementing these experiments in the field would require substantial additional infrastructure and resources. Importantly, we found that sleep patterns observed in the laboratory closely mirror those in the wild. This similarity supports the physiological relevance of the arousal threshold experiments conducted in the lab and suggests that lab-based findings are applicable to natural settings.

Comment:
Line 107-108: This sentence is clunky and could even be removed without loss of content.

Response:
These sentences have been combined and revised; the updated version now appears on Page 4, Line 124.

“In contrast to C. andromeda, under LD, N. vectensis showed rhythmic locomotor activity (Fig. 2c), increased between ZT6-ZT18 and decreased between ZT18-ZT6 (Fig. 2d).”

Comment:
Line 111. In the future, you should simulate dawn and dusk in the lab with transitions between light and dark.

Response:
We agree with the reviewer’s comment and plan to implement this in future studies. Indeed, the animals are highly sensitive to sleep–wake transitions. To better mimic their natural habitat, we will employ a lighting system that gradually transitions between light and dark conditions. This approach will allow us to monitor sleep while simulating the natural light environment. Based on

the consistent results observed in our lab and field experiments with jellyfish, we anticipate that sea anemones will exhibit similar behavior under both light conditions—excluding the one-hour period immediately following light–dark transitions.

Comment:
Line 121: You have characterized sleep, not defined it. Use of the word 'define' is circular.

Response:
As suggested by the reviewer, this sentence has been revised in Page 4, Line 116 to:

"…sleep was not characterized in sea anemones, including N. vectensis.

Comment:
Line 124: To look at circadian regulation, normally, one would do many photoperiodic regimes, including 12:12 LD, 24:0 LL, 0:24 DD but also 6:6:6:6 (LDLD) and others. The latter is called forced desynchrony.

Response:
In the original version of the manuscript, we had already conducted experiments under LD, LL, and DD conditions. We agree with the reviewer that introducing a desynchrony experiment (LDLD; 6L:6D:6L:6D) could provide deeper insight into how light and the circadian clock interact to regulate locomotor activity and sleep. As suggested, we therefore included new data in the revised manuscript, examining the behavior of *C. andromeda*, as well as wild-type (WT) and *NvClk*$^{\Delta/\Delta}$ mutant *N. vectensis*, under LDLD conditions:

- In *C. andromeda*, locomotor activity displayed a ~12-hour rhythm in LDLD (Extended Data Fig. 1e-g), indicating that behavior is primarily driven by the light/dark cycle rather than the circadian clock. This interpretation is consistent with the arrhythmic activity observed under both DD and LL conditions (Extended Data Fig. 1a-d).
- In contrast, WT *N. vectensis* maintained a robust ~24-hour locomotor rhythm in LDLD (Extended Data Fig. 3e,f), suggesting circadian control. This conclusion is further supported by two observations: (1) *NvClk*$^{\Delta/\Delta}$ mutants lost the ~24-hour rhythm under LDLD (Extended Data Fig. 3g-h), and (2) WT animals retained ~24-hour rhythmicity under both DD and LL (Extended Data Fig. 3a-d; Aguillon *et al.* 2024).
- Interestingly, LDLD also induced distinct sleep/wake patterns: ~12-hour cycles in WT *N. vectensis* and ~6-hour cycles in the *NvClk*$^{\Delta/\Delta}$ mutants. These findings indicate that while the circadian clock is the primary regulator of sleep, as evidenced by the ~24-hour sleep rhythm observed under DD (Fig. 2l-n), light/dark transitions also play a modulatory role in sleep regulation in N. vectensis.

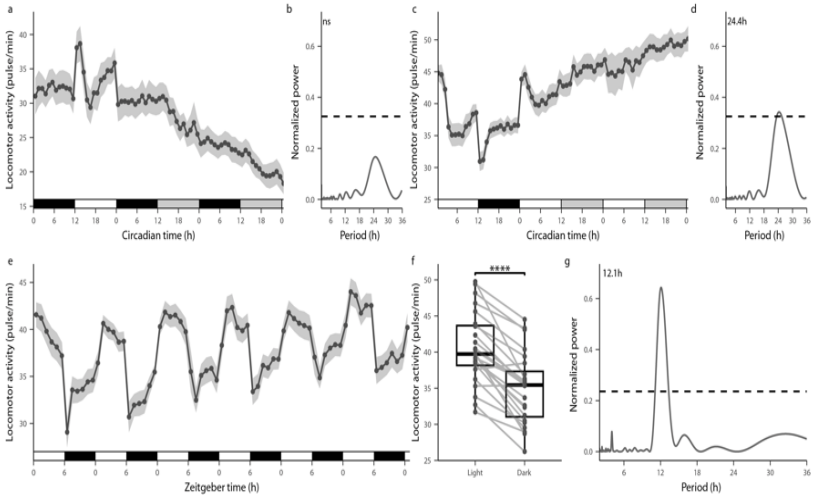
In the revised version of the manuscript, the LDLD results are presented in the Results section and illustrated in Extended Data Fig. 1 and Extended Data Fig. 2.

Page 3, Line 89: *"However, rhythmic locomotor activity was abolished under constant darkness (DD) and robustly dampened under constant light (LL) conditions (Extended Data Fig. 1a-d). Moreover, under forced desynchrony conditions (6h L:6h D:6h L:6h D, LDLD), the animals showed*

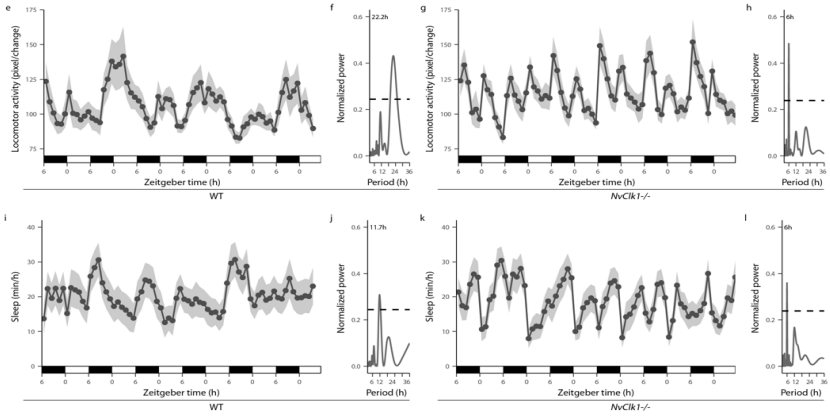
a 12.1h rhythm of locomotor activity (Extended Data Fig. 1e-g). These findings showed that sleep is primarily driven by the light/dark cycle in C. andromeda."

Page 5, Line 144: "To further differentiate between light- and circadian-regulation of sleep, we monitored sleep under forced desynchrony (LDLD, as in C. andromeda). While the rhythmic period of locomotor activity was 22.2h and controlled by the clock, sleep period was 11.7h (Extended Data Fig. 3e, f, i, j). These results suggest that although sleep is primarily regulated by the circadian clock it can also be modulated by light in N. vectensis. To understand the hierarchy of light- and intrinsic clock-regulation of sleep, we monitored sleep in NvClk$^{\Delta}$ mutant^{34} (NvClk$^{\Delta/\Delta}$) N. vectensis. NvClk$^{\Delta/\Delta}$ showed bimodal sleep/wake rhythms (periods of 12.25h and 25.24h) under LD and this rhythm was abolished under DD (Fig. 2o-q, Extended Data Fig. 3c, d). Under LDLD, the sleep/wake cycle followed the 6h alternating light/dark rhythm (Extended Data Fig.3k, l). These findings demonstrate that the transcription factor NvClk is essential for maintaining clock-controlled sleep in N. vectensis"

Extended Data Fig.1a-g



Extended Data Fig.2e-l



Comment:

Line 144. Please remove the term 'sleep quality' throughout. This term originates in humans with

the assumption that sleep continuity is the most important feature of sleep. In non-human animals, we have no idea what constitutes “high-quality” sleep or not. For instance, the recent report of 10,000 sleep bouts per day in penguins… is that high quality sleep or not? Ultimately, we have no idea, since the term is vague and best avoided. You could just say you measured the duration of sleep episodes.

Response:
As suggested by the reviewer, we have removed the term “sleep quality” and replaced it with “sleep architecture” throughout the manuscript.

Comment:
Line 154. Melatonin is only sleep promoting in diurnal species, thus it is not surprising it had little effect in crepuscular anemones. It is important to note also (line 158) that melatonin has already been shown to increase sleep in jellyfish. That said, you could say nocturnal flatworms given melatonin show reduced activity (Omond et al 2017).

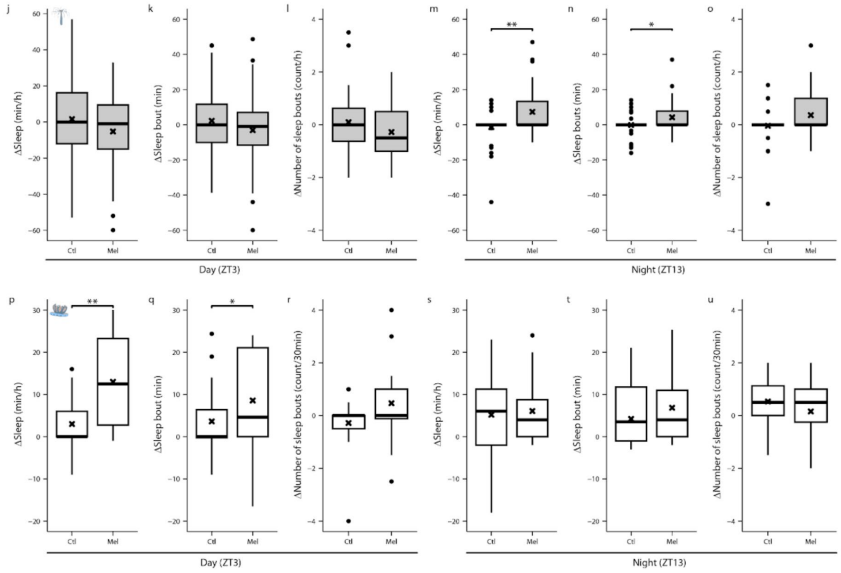
Response:
The differential effect of melatonin on sleep in nocturnal and diurnal animals remains controversial and inconclusive. Melatonin is a circadian marker of night and is presumed to promote sleep in diurnal animals and wakefulness in nocturnal animals. However, studies have shown that melatonin can promote sleep even in nocturnal mice during the night[2]. As noted by the reviewer, and in support of this observation, melatonin treatment was found to reduce nighttime activity in nocturnal flatworms[3].

In the first version of the manuscript, we assessed the effect of melatonin at a single time point. In the revised version, we expanded these assays to evaluate the effect of exogenous melatonin during both day and night in both species. In the diurnal *C. andromeda*, melatonin increased sleep only during the day (Fig.3p-r), while in the crepuscular *N. vectensis*, it promoted sleep only during the night (Fig.3m-o). In both species, melatonin had no observable effect during the natural sleep period, possibly because endogenous sleep masked the pharmacological impact (Fig.3j-l and s-u). These findings demonstrate a conserved sleep-promoting function of exogenous melatonin, irrespective of chronotype, and are consistent with previous observations in rats and flatworms.

The new melatonin-related data are now described in the Results, Methods, and Discussion sections of the revised manuscript. Page 06, Line 187: *“We tested the effect of melatonin on sleep in the diurnal C. andromeda and crepuscular N. vectensis. While melatonin treatment did not affect sleep during the rest periods of N. vectensis (ZT3) and C. andromeda (ZT13) (Fig. 3j-l, s-u), sleep time, sleep bout duration, and the number of sleep bouts increased during the active phase in both N. vectensis (ZT13) and C. andromeda (ZT3) (Fig. 3m-o, p-r). These results suggest an evolutionarily conserved role of melatonin in sleep regulation across animals with different chronotypes”* Page 09, Line 298: *“…despite their opposing chronotypes, melatonin treatment promoted sleep in both species during their respective active phases, suggesting a conserved role for this hormone in sleep modulation.”* Page 22, Line 646: *“Both species received either 100 μM*

melatonin (diluted in ethanol) or an ethanol control at ZT3 and ZT13, corresponding to their respective periods of wakefulness and activity."

These new results are now shown in Fig.3j-u:



Comment:
Line 233. I think the work Symbiodiniaceae can go since you refer to them first as dinoflagelattes.

Response:
As recommended by the reviewer, the word "Symbiodiniaceae" has been deleted in page 09, line 287: *"The light-dependence regulation may be attributed to the C. andromeda symbiosis with endosymbiotic dinoflagellates ."*

Comment:
Line 281-283. I think these last lines would benefit from expansion, even while speculative. All life is made of DNA and much of that life is transparent, microscopic, sea-living and exposed to sunlight rich in UV and X-rays. I'd like to hear more on the authors thoughts on where they think sleep began.

Response:
Following the reviewer's suggestion, we have expanded the Discussion, which now reads as follows (page 11, line 350):

"Moreover, considering that the interplay between the circadian clock and genomic stability has been shown in non-neuronal mammalian cultured cells, fungi and cyanobacteria[78–81], sleep-like states may have preceded the evolution of neurons, originally evolving to support synchronized and consolidate periods of cellular maintenance."

Reviewer #2 (Remarks to the Author):

This paper represents the third reported instance of sleep phenomena in cnidarians, might be contributing significantly to the clarification of the evolutionary discourse on sleep. I assume the identification of sleep phenomena in both *C. andromeda* and *N. vectensis* likely demanded considerable effort. Moreover, beyond the mere reporting of sleep phenomena, the paper delves into the significance of sleep, noting the observed relationship between DNA damage and sleep in cnidarians, echoing findings in other organisms and addressing the early significance of sleep in animals.

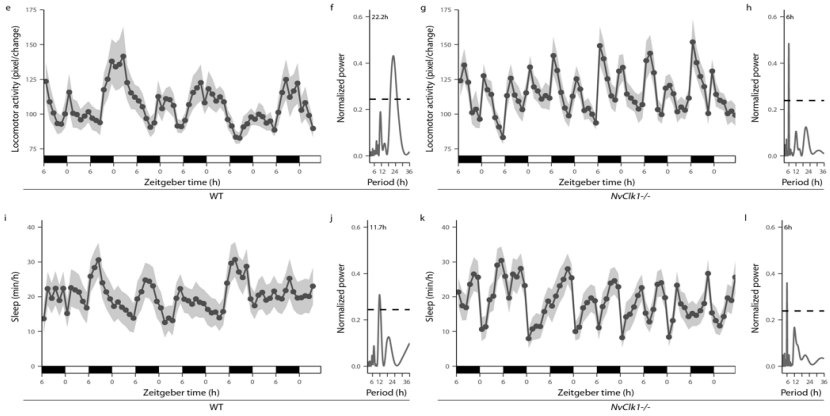
However, given the significance of the research and its findings, meticulous attention to the paper's structure, methodology, analysis, statistics, and figure presentation is warranted. Particularly, the presence of notation in the statistics section that may not be readily understandable is regrettable. It is imperative that the presentation be crafted to allow readers to grasp the content instantaneously and clearly. Additionally, some roughness is evident in the experimental aspect concerning the relationship between DNA damage and sleep, prompting cautious consideration of the paper's claims. Below, I have enumerated specific concerns that stand out, suggesting that addressing these issues is essential at the very least.

Major:

Comment:
1. The vertical axis of Fig.1c does not start from zero, giving the impression of a clear LD cycle, but in reality, the difference in pulses per minute is very small. The increase in activity immediately after the light period and before the dark period suggests that much of the difference between day and night in Fig1.d is due to this rise in activity. This bimodal activity, also observed in organisms like Drosophila, often signifies time recognition by the circadian clock. However, as the authors themselves note, based on the results in Extended Data Fig. 1a-d, it seems unlikely that a circadian rhythm is controlling behavior in *C. andromeda*. In that case, how should we interpret the bimodal pattern seen in Fig1.c? Given its likely significant impact on the difference between day and night in Fig1.d, it should be adequately explained. For instance, it would be advisable to investigate what activity and sleep cycles are observed when the LD cycle is changed to 6h:6h or 3h:3h instead of 12h:12h.

Response:
We thank the reviewer for this insightful mechanistic comment. In Fig.1c, the average sleep time of the population never reached zero; therefore, we displayed the relevant range of data on the Y-axis. We agree that the difference in pulsation rate between light and dark periods is relatively small. However, this difference is consistently significant in both laboratory and field settings—even under gradual light/dark transitions—and is highly relevant to the animal’s physiology, as demonstrated in other figures. As noted by the reviewer, monitoring behavior under 12 h light:12 h dark (LD), constant darkness (DD), and constant light (LL) suggests that the bimodal activity observed in *C. andromeda* is primarily light-driven. Additionally, our sleep deprivation experiments

indicate that sleep regulation involves a homeostatic component. To further support this mechanism, as suggested by the reviewer, we conducted a 6L:6D:6L:6D forced desynchrony experiment in the revised manuscript. The results clearly show that behavior is primarily regulated by the light/dark cycle. This new data is presented in Extended Data Fig. 1e–g.



This data has been integrated into the Results section (page 06, line 144): "*To further differentiate between light- and circadian-regulation of sleep, we monitored sleep under forced desynchrony (LDLD, as in C. andromeda). While the rhythmic period of locomotor activity was 22.2h and controlled by the clock, sleep period was 11.7h (Extended Data Fig. 3e, f, i, j). These results suggest that although sleep is primarily regulated by the circadian clock it can also be modulated by light in N. vectensis. To understand the hierarchy of light- and intrinsic clock-regulation of sleep, we monitored sleep in NvClk$^{\Delta}$ mutant^{34} (NvClk$^{\Delta/\Delta}$) N. vectensis. NvClk$^{\Delta/\Delta}$ showed bimodal sleep/wake rhythms (periods of 12.25h and 25.24h) under LD and this rhythm was abolished under DD (Fig. 2o-q, Extended Data Fig. 3c, d). Under LDLD, the sleep/wake cycle followed the 6h alternating light/dark rhythm (Extended Data Fig.3k, l). These findings demonstrate that the transcription factor NvClk is essential for maintaining clock-controlled sleep in N. vectensis".* In addition, we add this sentence to the discussion (page 09, line 285): *"Light and homeostatic drive were the predominant sleep regulatory processes in C. andromeda, while homeostatic drive and the circadian clock control sleep in N. vectensis.".*

Comment:

2. In Figures 1o and p, it is not easy to find explanations regarding sample size and statistics, making it difficult to properly evaluate the results. To assert the validity of the results, descriptions regarding sample size and statistics should be made explicit.

Response:

We thank the reviewer for the insightful comment. In the revised manuscript, we have explicitly added the sample size and statistical information as suggested. Specifically, we have updated the figure legends to make these details clear and easily accessible. For example: Fig.1o and p, page 25, line 716: "*(o) Sleep percentages in control (Ctl; n=14) and sleep-deprived (SD; n=18) animals under LD. (p) Average percentage of sleep in Ctl and SD animals during the day and night pre-*

SD, and during the day post-SD." And page 25 line 725: *"For panel (p): Tukey HSD (post-hoc analysis following ANOVA), letters indicate significant differences ($p < 0.05$)."*

Comment:
3. The mentioned statement, particularly "36.4 pulses/min, Fig. 1q", refers to a specific data point in Figure 1q where the average pulse rate during the day at sea is represented. This data point is crucial for understanding the comparison being made in the sentence. Additionally, it is indeed conceivable that locomotor activity (measured in pulses/min) might be influenced not only by bell size but also by temperature variations. Therefore, it would be prudent to discuss the potential impact of seawater temperature (changes) on locomotor activity.

Response:
The value of 36.4 pulses per minute cited in the manuscript represents the average daytime pulse rate observed in the laboratory (Fig. 1d), while the threshold of 37 pulses per minute was set to distinguish between sleep and wake states (Fig. 1f–h). Given the close similarity between these values, we approximated the sleep threshold based on the laboratory-derived average to estimate sleep time in the field across animals of varying sizes (Fig. 1q–s). This clarification has been added to the Results section (Page 04, Line 106): *"Notably, the pulsation threshold used to detect sleep under controlled laboratory conditions (37 pulses/min, Fig. 1f-h) closely matched the average daytime pulse rate observed in the lab (36.4 pulses/min, Fig. 1d). Given this similarity, we approximated the sleep threshold in the field by the average daytime pulse rate, adjusted according to the bell diameter, as a proxy to differentiate sleeping and awake animals."*

Despite the environmental differences between the lab (Bar-Ilan University, Israel; 24 °C, 35 ppt salinity) and the field (Key Largo, FL, USA; 26.8 °C, 31.5 ppt salinity), sleep patterns in *C. andromeda* were consistent between day and night. The animals predominantly slept during the night. Intriguingly, under both lab and field conditions, we observed a siesta-like sleep phase around midday (Fig. 1i, s). This consistency suggests that while seawater temperature and salinity may influence the absolute level of locomotor activity, the overall sleep pattern is robust and primarily driven by the light/dark cycle[8].

To emphasize this point, we added the following sentence to the revised manuscript (Page 09, Line 281): *"These animals exhibited similar sleep duration and architecture as in controlled laboratory conditions, including short midday naps despite differences in salinity and temperature."*

Comment:
4. In the experiments presented in Fig.2f-k, the latency to response when stimuli were applied to individuals in an active state cannot be considered as a response time. Therefore, the latency to response when the Length of quiescent bout is 0 should be excluded. Its inclusion would naturally result in shorter response times when the quiescent bout is less than 8 minutes, significantly affecting the definition of sleep. Especially considering that Fig.3 also measures bout length, there is concern that this inclusion may influence other results as well.

Response:
We thank the reviewer for pointing this out. Upon re-examining our analysis, we identified an error introduced by the use of the jitter function in R. In the revised version of the manuscript, we have corrected this error, which led to slight changes in Fig.2f–k.

Original Fig.2f–k (initial submission)

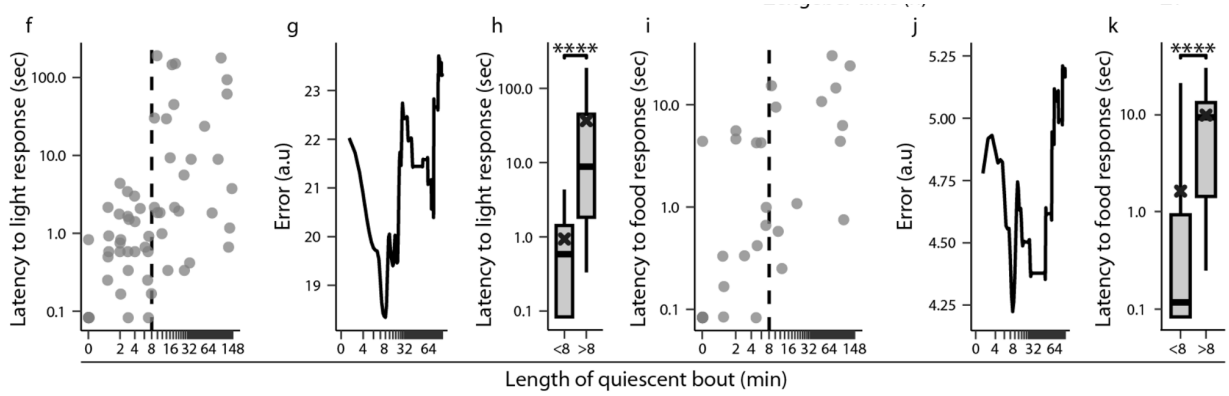
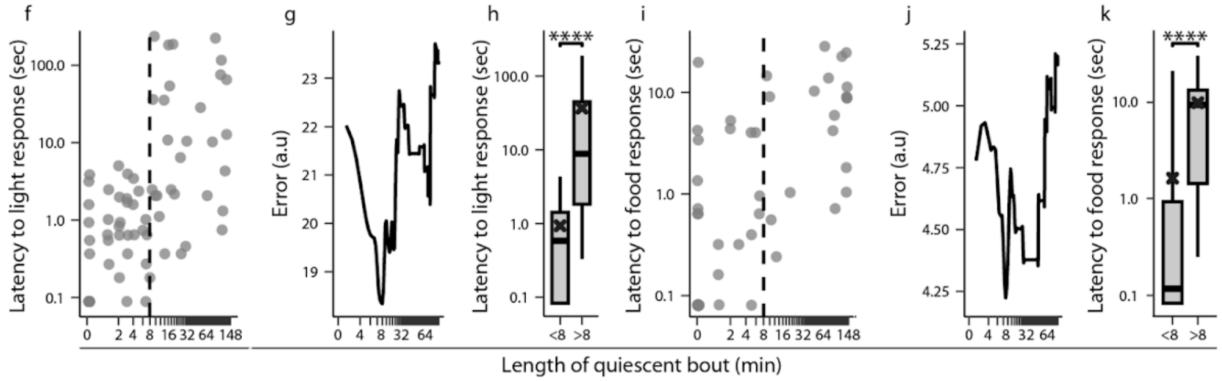


Fig.2f-k in the revised version:



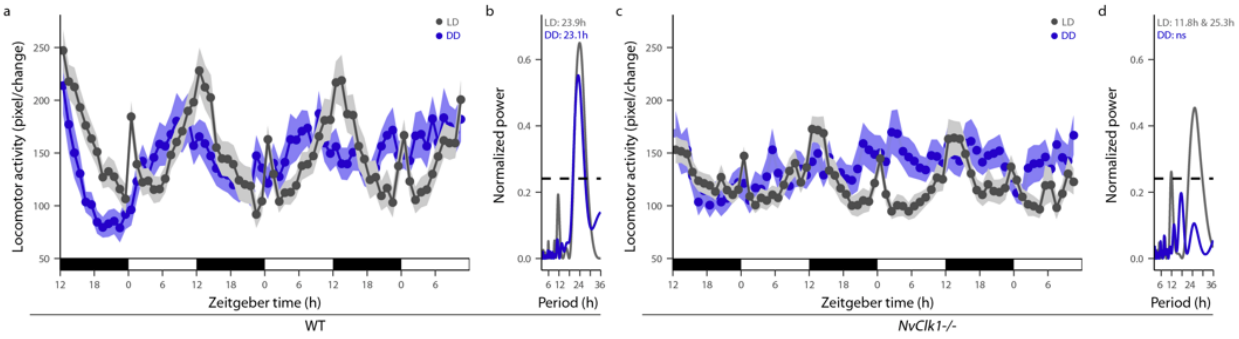
Furthermore, as recommended by the reviewer, we excluded animals that were active at the time of stimulus onset. This exclusion did not alter the threshold value of 8 minutes used to identify sleeping animals.

Comment:
5. In Fig2.l-q, if discussing the sleep patterns of DD and NvClk mutant, it would be beneficial to include their activity rhythms as well. This would enhance clarity and facilitate better understanding of the data.

Response:
We agree with the reviewer that including activity rhythms alongside the sleep data would enhance the clarity and interpretation of the results. In the revised version of the manuscript, we have added the locomotor activity rhythms of both WT and *NvClk*$^{\Delta/\Delta}$ *N. vectensis* (Extended Data Fig. 3a-d), which now complement the sleep data presented in Fig. 2i-q.

To describe these data, we added the following text to the Results section (Page 05, Line 139): *"Similarly, under DD the rhythmic sleep period was 22.5h (Fig. 2l-n, Extended Data Fig. 3a, b), suggesting that sleep is regulated by the circadian clock in N. vectensis. To further differentiate between light- and circadian regulation of sleep, we monitored sleep under forced desynchrony (LDLD). While the rhythmic period of locomotor activity was 22.2h and controlled by the clock, sleep period was 11.2h (Extended Data Fig. 3e, f, i, j)."*
"NvClk$^{\Delta/\Delta}$ showed bimodal sleep/wake rhythms (periods of 12.25h and 25.24h) under LD and this rhythm was abolished under DD (Fig. 2o-q, Extended Data Fig. 3c, d)."

Extended Data Fig. 3A-D:



Comment:
6. In the experiments presented in Fig.2r, and s, how were the close tentacle and the open tentacle distinguished from the imaging data? Is it possible that the difference in sleep quantity between them is due to the detection of tentacle movement as activity, leading to an underestimation of sleep?

Response:
The open or closed tentacle state was determined manually. Animals were categorized as 'open' if they displayed more than two visible tentacles. Notably, while open and closed tentacle postures are associated with wake and sleep states, respectively, they are not definitive indicators of behavioral state; for example, animals with closed tentacles can be awake, and those with open tentacles can be asleep. To quantify activity, we assessed the movement of the entire body rather than relying solely on tentacle or body column motion, similar to approaches used in Hydra. Our data indicate that tentacle posture alone is not a reliable determinant of sleep (Fig. 2r, s). Nonetheless, in future studies, a more detailed analysis of body part-specific movements may help refine the characterization of sleep and potentially uncover sleep stages.

To clarify the methodology for defining the open versus closed tentacle state, we added the following text to the Methods section (Page 22, Line 638*): "Open or closed posture was manually annotated from a single video frame. Animals were categorized based on the number of tentacles deployed outside of their body. Animals that showed at least two visible tentacles were classified as 'open'."*

Comment:
7. The statistics regarding the results in Fig.2t, and u are not readily apparent, making it difficult to evaluate the results properly. While it is possible that they are mentioned somewhere, it is not immediately clear, so please ensure that they are presented clearly for easy understanding.

Response:
As requested, we clarified the statistical details. In the revised version of the manuscript, Fig. 2t and Fig. 2v now include visual indicators highlighting the time windows selected for the subsequent statistical analyses shown in Fig. 2u and Fig. 2w. The statistical details are explicitly provided in the legend of Fig. 2, page 26 line 755: *(t) Average sleep time in control (Ctl: n=22) and sleep-deprived (SD: n=24) animals during the day and night before and after SD (ZT20-ZT3). The time window (ZT10-16) used to compare post-SD sleep time in panel (u) is indicated above the curves. (u) Average sleep time in Ctl and SD animals at ZT10-16 (Ctl: n=22, SD: n=24). (v) Average sleep time in control (Ctl: n=24) and sleep-deprived (SD: n=28) animals before and after SD (ZT8-ZT15), serving as a mirror control for SD experiment in panel (t). The time window (ZT8-ZT15) used for comparison in panel (w) is indicated above the curve. (w) Average sleep time in Ctl and SD animals at ZT8-ZT15 (Ctl: n=24, SD: n=28)….For panels (h), (k), (t), (u), (w): Mann Whitney Wilcoxon... ****p<0.0001"* In addition, we have revised the Results section accordingly (Page 05, Line 161): "*To test whether homeostatic process regulates sleep, we sleep-deprived the N. vectensis for 8h during sleep period (ZT20-ZT3) using gradual and gentle water exchange once per hour (Extended Data Movie 4). Following SD, sleep-deprived animals slept 1.24-fold more than control animals (Fig. 2t, u), indicating a homeostatic sleep drive. In contrast, control SD during the activity period (ZT8-ZT15) did not induce a sleep rebound (Fig. 2v, w)."*

Comment:
8. In Fig.3c, e, and g, why are mean transitions presented as count per hour? In this case, it seems that representing the number of bouts per 12 hours would be the simplest and most basic way of notation.

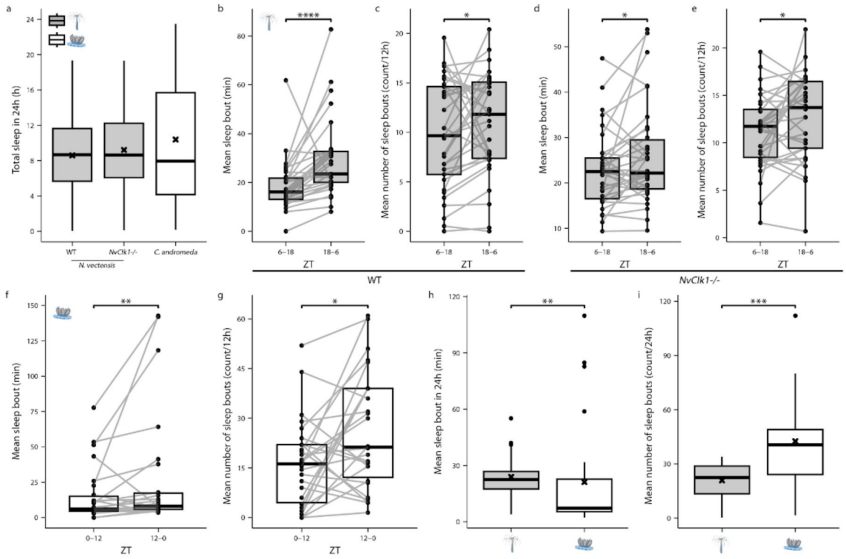
Response:

We chose to present transitions as counts per hour to maintain consistency with the behavioral analyses shown in Figures 1 and 2. However, we agree with the reviewer that presenting the number of bouts per 12-hour period offers better clarity for the reader. Accordingly, we reanalyzed the data and quantified the number of sleep bouts and state transitions as follows:

- During the analysis window, any sleep bout already ongoing at the end of the window was counted as half a bout.
- In contrast, a complete bout—one that both began and ended within the time window—was counted in full.
- This adjustment was necessary because a complete bout consists of two transitions, whereas an incomplete bout includes only one.
- This weighting method allows us to preserve information on sleep fragmentation while converting the data into sleep bout counts.

The revised data are now presented in Figure 3 and are described in the Results section (Page 06, Line 173): *"To determine sleep patterns, we quantified the length and number of sleep bouts during the sleep and wake periods of each species. We found longer sleep bout durations and a higher number of sleep bouts at ZT18-6 compared to ZT6-18, under LD, in both WT (Fig. 3b, c) and NvClk$^{D/D}$ (Fig. 3d, e) N. vectensis. Similarly, under LD conditions, sleep bout length and the number of sleep bouts increased during the night (ZT12-0) in the diurnal C. andromeda (Fig. 3f, g). Inter-species comparison of sleep architecture revealed that although total sleep time was similar (Fig. 3a), the average sleep bout length was shorter by 2.8min (Fig. 3h), and the mean number of sleep bouts was approximately two-fold higher (Fig. 3i) in C. andromeda compared to N. vectensis. These results show that, like humans, both cnidarian species spend approximately one-third of their lives sleeping (Fig. 3a). However, sleep is more consolidated in N. vectensis, i.e., longer and less fragmented sleep bouts, compared to C. andromeda."*

Fig. 3a-i



Comment:

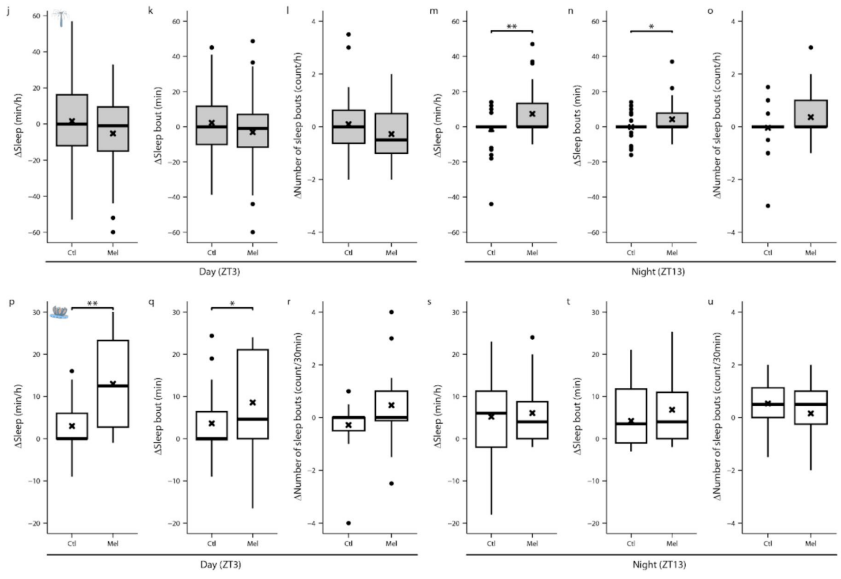
9. In Fig3j, kl, and m, as melatonin is known to influence circadian rhythms, it is important to show the sleep patterns when melatonin is added, not only to determine its effect on sleep but also to clearly distinguish its influence from that of circadian rhythms. Additionally, I feel a slight discomfort with the fact that detailed analyses such as day versus night or ZT6-ZT18 versus ZT18-ZT6 have not been conducted, which would provide a comparison with previous analyses.

Response:

In response to the comments of this and other reviewers, we extended the melatonin treatments to include an additional time window. A limitation of long-term pharmacological experiments is that the removal of melatonin—typically achieved through water exchange—can itself stimulate animal behavior, as demonstrated in our sleep deprivation protocol. This stimulation may mask the effects of the hormone, complicating interpretation. To detect the long-term circadian effect, a different behavioral set up is required including a continuous water flow alternative method of sleep

detection. Nevertheless, as suggested by the reviewer and to assess the differential effects of melatonin during the day and night, we quantified sleep time and architecture during the active (ZT3 for *C. andromeda*; ZT13 for *N. vectensis*) and sleep (ZT13 for *C. andromeda*; ZT3 for *N. vectensis*) periods. The results showed that melatonin promotes sleep during the active period in both crepuscular and diurnal cnidarians, supporting the notion that melatonin promotes sleep regardless of the animal's chronotype. Similar effects have also been reported in other diurnal and nocturnal species[2,3,9].

The new melatonin-related data are now described in the Results, Methods, and Discussion sections of the revised manuscript. Page 06, Line 187: *"We tested the effect of melatonin on sleep in the diurnal C. andromeda and crepuscular N. vectensis. While melatonin treatment did not affect sleep during the rest periods of N. vectensis (ZT3) and C. andromeda (ZT13) (Fig. 3j-l, s-u), sleep time, sleep bout duration, and the number of sleep bouts increased during the active phase in both N. vectensis (ZT13) and C. andromeda (ZT3) (Fig. 3m-o, p-r). These results suggest an evolutionarily conserved role of melatonin in sleep regulation across animals with different chronotypes"* Page 09, Line 298: *"…despite their opposing chronotypes, melatonin treatment promoted sleep in both species during their respective active phases, suggesting a conserved role for this hormone in sleep modulation."* Page 22, Line 646: *"Both species received either 100 μM melatonin (diluted in ethanol) or an ethanol control at ZT3 and ZT13, corresponding to their respective periods of wakefulness and activity."*



Comment:

10. The figures in Fig. 4a and b are difficult to understand immediately, so please make efforts to create figures that are easier for readers to comprehend. It is unclear why, despite both graphs having the same vertical axis labeled as 'average sleep time during 12h preceding each time point,' the range of transitions in (a) is approximately 40% to 60%, while in (b) it remains between 15% and 30%.

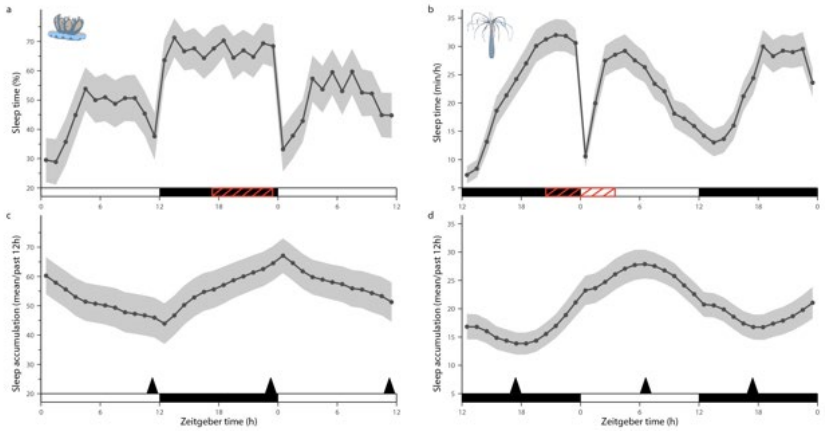
Response:

Thank you for the helpful feedback. To improve clarity and make the charts in Fig. 4c and 4d easier to interpret, we have added average sleep time charts (Fig. 4a and 4b) above the corresponding sleep accumulation charts. The data presented in Fig. 4c and 4d are derived directly from the sleep time data shown in Fig. 4a and 4b, respectively.

The differences in the Y-axis between Fig. 4c and Fig. 4d reflect the distinct units used to quantify sleep: percentage for *C. andromeda* and hours for *N. vectensis*. This dataset-specific scaling facilitates the visual identification of transitions between sleep and wake states.

These changes are now described in the Results section, page 07, Line 201: *"We hypothesized that DNA damage would peak and decline following extended wake and sleep, respectively. To identify the time points of maximal and minimal sleep pressure, we calculated the average sleep time over the past 12h that preceded each time point. Both C. andromeda and N. vectensis were sampled at the end of their wake (ZT11 and ZT17) and sleep (ZT23 and ZT8) phases (Fig. 4a, b), where wake and sleep accumulation peaked (Fig. 4c, d)."*

In addition, we modified the units in the Y axis and presented it in Fig. 4:



Comment:
Additionally, the statistics regarding the results in Fig.4g and h are not readily apparent, making it difficult to evaluate the results properly. While they may be mentioned somewhere, it is not immediately clear, so please ensure that they are presented clearly for easy understanding.

Response:
Thank you for the comment. As explained above, and in accordance with Nature Communications guidelines, we have indicated the statistical information for Fig. 4i and 4j (previously Fig. 4g and 4h) in the corresponding figure legend. In the revised manuscript we modified the statistical details presentation and provided a clear statistical summary at the end of each figure legend. Letters are used to denote statistically significant differences. This is now indicated in the relevant legend,

such as in Fig. 4 legend, page 27, line 813: *"In (I, j) different lowercase letters above the bars indicate statistically significant at p<0.05".*

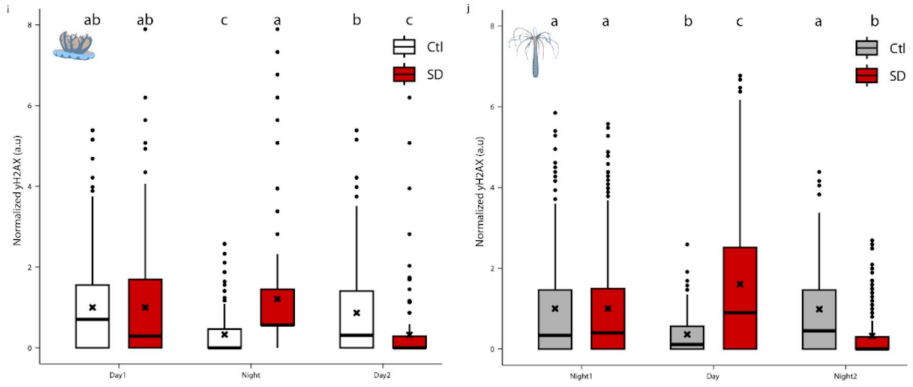
Comment:
11. The figures in Fig. 4g and h exhibit a considerable number of outliers, giving the impression that the discussion is based on very marginal differences. Therefore, I believe there might be room for improvement in the analysis method or experimental conditions. If the authors' claims regarding the experimental results in Fig. 4 are accurate, providing continuous sleep deprivation (SD) could potentially lead to a higher accumulation of DNA damage. Conducting long-term SD experiments might enhance the credibility by demonstrating clearer differences.

Response:
Thank you for this insightful comment, which inspired us to develop a more efficient sleep deprivation (SD) protocol. The observed variability in DNA damage is expected, given the distinct identities, activity levels, and functional roles of individual neurons. Similar heterogeneity has been reported in other model organisms[10–12], and such assays typically require relatively large sample sizes for robust interpretation.

To overcome these limitations and enhance the impact of SD on DNA damage, we implemented an improved SD protocol in the revised manuscript. *N. vectensis* were sleep-deprived using hourly water flow stimulation, a method that proved more effective and minimized circadian interference compared to light-based stimulation. SD administered during the natural sleep phase successfully induced wakefulness, followed by a clear sleep rebound the subsequent night (Fig. 2t–w). Correspondingly, DNA damage levels increased following SD (ZT8) and decreased after the sleep rebound period (ZT17). These results support the conclusion that sleep plays a protective role in alleviating DNA damage.

These findings are detailed in the Results section (Page 07, Line 210): *"Under LD, the number of γH2AX foci increased at the end of the wakefulness period in C. andromeda (ZT11) and N. vectensis (ZT17) and reduced at the end of the sleep period in C. andromeda (ZT23) and N. vectensis (ZT8) (Fig. 4i, j). The opposite timing of DNA damage reduction between C. andromeda and N. vectensis was aligned with their species-specific chronotype and daily timing of sleep. To determine the effect of homeostatic sleep pressure, the C. andromeda and N. vectensis were sleep-deprived for 6h (ZT17-ZT23) and 8h (ZT20-ZT4), respectively. Following SD, the number of γH2AX foci increased significantly in C. andromeda (ZT23) and N. vectensis (ZT8) and subsequently decreased following sleep recovery (Fig. 1o, p; Fig. 2t, u) in both species (Fig. 4i, j)."* In addition, we explained the SD protocol in the methods section page 22, line 634: *"In SD experiments, the animals were stimulated by gentle water exchange once per hour during their peak of sleep period (ZT20-ZT4) and peak of activity period (ZT8-ZT16; Extended Data Movie 4) and sleep was monitored before and after the SD."*

We also modified Fig.4i, j:



Comment:

12. It is difficult to determine whether DNA damage is involved in sleep based solely on the experimental method in Fig. 5. Changes in locomotor activity due to UV irradiation should also be included. Additionally, observing the relationship between the number of γH2AX foci on the day after UV irradiation and two days after UV irradiation and sleep would provide a more accurate discussion of the relationship between DNA damage and sleep.

Response:

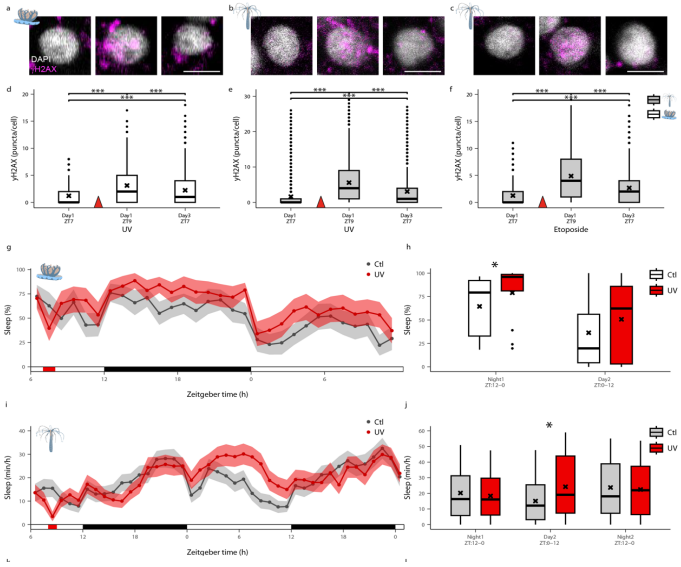
We agree with the reviewer that continuous behavioral monitoring over multiple days is essential to determine the effects of UV-induced DNA damage on sleep. As requested, we monitored sleep in UV-treated and control animals for 30 hours in *C. andromeda* and 38 hours in *N. vectensis*. In parallel, we imaged the animals and quantified DNA damage in individual neurons at multiple time points: before UV exposure (ZT7), immediately after UV irradiation (ZT9), and during the recovery period (ZT3, two days later). Notably, UV irradiation led to increased DNA damage (Fig. X). During the subsequent sleep phase—nighttime in *C. andromeda* and daytime in *N. vectensis*—we observed a significant increase in sleep (Fig. 5g-j). Remarkably, in both species, this sleep recovery period was followed by a reduction in DNA damage levels. These findings support the hypothesis that DNA damage induces sleep pressure and that sleep facilitates DNA repair.

These results are described in the Results section (Page 08, Line 229): *"In C. andromeda and N. vectensis, the number of γH2AX foci increased one hour after UV exposure (Fig. 5a, b, d, e). Subsequently, nighttime and daytime sleep increased in C. andromeda (Fig. 5g, h) and N. vectensis (Fig. 5i, j), respectively. Notably, following sleep recovery, the animals remained viable, and two days later the levels of DNA damage reduced in both species (Fig. 5a, b, d, e). These results suggest that UV-mediated induction of DNA damage increases sleep pressure."*

The experimental protocol is detailed in the methods section (page 23, line 652): *"C. andromeda were habituated for 72h and entrained to an LD cycle. A plastic barrier divided and isolated UV-treated animals from control in the behavioral monitoring system. Sleep was recorded during 30h and following 1h (ZT8) half of the C. andromeda were exposed to 15min 312nm UVB light (Spectroline® E-Series UV lamp) located 20cm above the animals. Similarly, N. vectensis were habituated for 3 days and entrained to LD cycle in six-well plates. Sleep was recorded for 42h, and following 2h, half of the N. vectensis were exposed to 1h of 312nm UV light (Spectroline® E-Series UV lamp), located 20cm above the animals. To quantify DNA damage, at each time point,*

5-7 UV-treated and control C. andromeda and N. vectensis were fixed in 4% Paraformaldehyde (PFA) before (ZT7), 1h (ZT9), and 48h (ZT7 Day2) following the UV irradiation."

The new data is presented in Fig. 5:



Minor:

Comment:
1. In LL and DD conditions, periodic activity is not observed in *C. andromeda* (Extended Data Fig. 1a-d). Does this suggest that clock genes are not present in the genome? Additionally, in LL conditions, the pulse/min is extremely high at the initial LD stage. Moreover, the sample size is n=6, smaller compared to others, raising some doubts about the reliability of these results. Please explain the black triangles in Fig. 1e represents, as it is unclear.

Response:
As recommended by the reviewer, and to validate our results, we repeated the constant light (LL) experiment with an independent cohort of animals (N = 16). We found that *C. andromeda* maintains a weak 24-hour rhythm (Lomb-Scargle Periodogram, LSP) under LL conditions. The pulse rate (pulses/min) during the initial LD phase of the experiment (Extended Data Fig. 1c) closely resembled that observed in the primary LD experiment (Fig. 1c). However, under LL, the rhythmicity was only marginally significant and was largely driven by the first cycle following the transition from LD to LL (Extended Data Fig. 1c, d).

Considering the arrhythmic behavior observed under both free-running conditions (DD and LL; Extended Data Fig. 1a–d), in contrast to the robust rhythms recorded under LD (Fig. 1c) and LDLD (Extended Data Fig. 1e), we conclude that rhythmic pulsation activity in *C. andromeda* is primarily regulated by external light/dark cycles rather than by an endogenous circadian clock.

Importantly, the absence of behavioral rhythms under constant conditions does not preclude the presence of circadian clock genes in the genome. Other rhythmic outputs—molecular, cellular, or

physiological—may still be under circadian regulation. Motivated by the reviewer's intriguing suggestion, we searched for circadian clock gene orthologs in the genome of *C. xamachana* (as the *C. andromeda* genome is currently unavailable) using the protein sequences of known *N. vectensis* clock genes. We identified several candidate orthologs:

NvCLK: Two bHLH-PAS domain-containing proteins (Casxa1/23723 and 23306) with 28.2% and 27.3% identity;

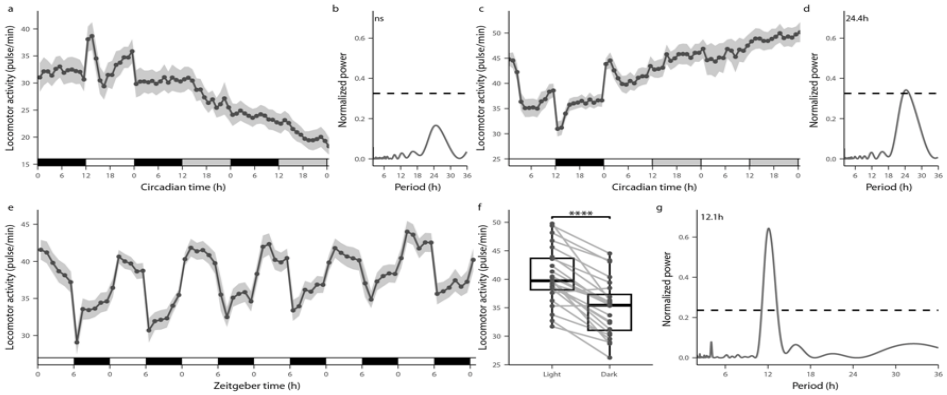
NvCYCLE: Five bHLH-PAS proteins (Casxa1/20122, 14058, 859, 1772, 23306) with identities ranging from 33% to 47%;

NvCRYa: Two cryptochrome proteins (Casxa1/27641 and 6769) with 51.3% and 36.1% identity.

These putative orthologs suggest that core circadian clock components are conserved in the *Cassiopea* genus. Further research will be necessary to determine their functional roles in regulating rhythmic processes.

In the revised manuscript, we described the conclusions of the LL experiment in the Results section (Page 03, Line 89): *"However, rhythmic locomotor activity was abolished under constant darkness (DD) and robustly dampened under constant light (LL) conditions (Extended Data Fig. 1a-d). Moreover, under forced desynchrony conditions (6h L:6h D:6h L:6h D, LDLD), the animals showed a 12.1h rhythm of locomotor activity (Extended Data Fig. 1e-g). These findings showed that sleep is primarily driven by the light/dark cycle in C. andromeda."* We also discuss these results in the discussion section (page 09, line 285): *Light and homeostatic drive were the predominant sleep regulatory processes in C. andromeda, while homeostatic drive and the circadian clock control sleep in N. vectensis."* In addition, we clarified the meaning of the black triangles in Fig. 1e by adding an explanation to the Methods section (Page 21, Line 594): *"A response was defined as a reduction of 2 standard deviations in the inter-pulse intervals (IPI) compared to the distribution of IPIs before the stimulation, within a time window of 10s after the stimulus."*

Extended Data Fig. 1a-g:



Comment:
2. Since the term 'optimization algorithm' may not be easily understood by general readers, could you provide a more specific description for clarity?

Response:

As requested we clarified this term. In the result section, we added in page 03 line 82 *"A threshold-based optimization algorithm was used to determine the optimal behavioral parameters to distinguish between slow and fast responses (Fig. 1g, j)."* and in the method page 21 line 596 *"A threshold-based optimization algorithm was used to determine the optimal behavioral parameters to distinguish between slow and fast responses (i.e., elevated arousal threshold; Fig. 1g, j). For each candidate threshold (e.g., pulsation rate), responses were split into two groups: below and above the threshold"*

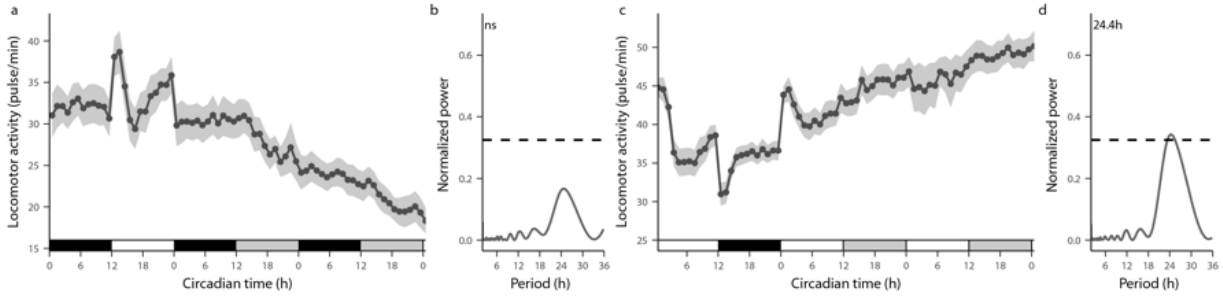
Comment:
3. In LL and DD conditions, periodic activity is not observed in *C. andromeda* (Extended Data Fig. 1a-d). Does this suggest that clock genes are not present in the genome? Additionally, in LL conditions, the pulse/min is extremely high at the initial LD stage. Moreover, the sample size is n=6, smaller compared to others, raising some doubts about the reliability of these results.

Response:
This comment duplicates Minor Comment 1. Please refer to our response above for a detailed explanation.

Comment:
4. In Extended Data Fig. 1c, it should be described as 'Circadian time (h)' under constant conditions. Additionally, Extended Data Fig. 1f should be described as 'time of day (h)' instead of 'Zeitgeber time (h).'

Response:
Thank you for the feedback. The 36-hour experiment consists of a 12 h:12 h light–dark (LD) cycle followed by constant light (LL). We acknowledge that the X-axis terminology may not have been clear. Following the reviewer's suggestion, we replaced "Zeitgeber time (h)" with "Circadian time (h)" in Extended Data Fig. 1c, and added "Time of day (h)" to Fig. 1s and Extended Data Fig. 1k.

These annotations have also been added to Extended Data Fig. 1a-d.



Comment:
5. I do not understand the meaning of the red dashed lines in Extended Data Fig. 2f-k. It is necessary to describe them more clearly for better understanding.

Response:
The red dashed line represents the threshold used to distinguish a behavioral response from quiescence. As noted by the reviewer, this was not explicitly indicated in the original figure legend. In the revised version, we have added the following explanation to the legend of Extended Data Fig. 2 (Page 30, Line 887): *"The red horizontal dashed line indicates the movement threshold."*

Comment:
6. L131: The text states 'ZT0-ZT8,' indicating an 8-hour sleep deprivation period during the day, while the legend mentions 'ZT0-ZT7.' To avoid confusion, it should be clarified which one is correct.

Response:
The correct time frame is "ZT0–ZT8," which corresponds to an 8-hour sleep deprivation (SD) period. However, following other reviewer recommendations, we revised the SD protocol to improve its effectiveness. Specifically, we applied gentle hourly water exchanges for 8 hours during the following time windows: ZT20–ZT4 and ZT8–ZT16.

Reviewer #3 (Remarks to the Author):
This manuscript examines the link between DNA damage in neurons and sleep, extending previous observations that were made in zebrafish and rodent models to more "ancient" sleepers, the jellyfish and anemone. Overall, the work is intriguing and is suggestive of a link between sleep and DNA damage even in these animals that lack brains. In particular, the work demonstrating a sleep-like state in the anemone is well done (with a few minor gaps, see minor comments) and the jellyfish behavioral work is a nice confirmation and extension of Nath et al., 2017. However, although problems with the data presentation and analysis of the DNA damage data makes it a little difficult to evaluate fully, the results don't seem to line up with the major hypothesis that DNA damage is resolved during sleep in these species, and in fact some of the data seems to contradict that notion entirely.

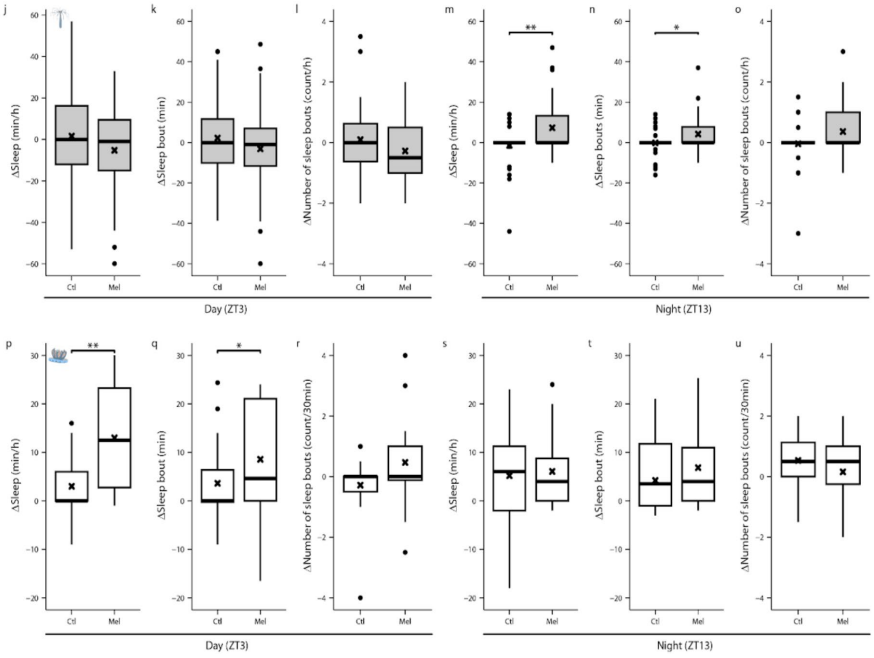
The first half of the manuscript, which describes the sleep state in jellyfish and the anemone is done to a high standard, and all the major expected behavioral criteria are examined (e.g. circadian period, sleep deprivation and recovery, arousal threshold changes). This makes for a convincing case that the two species have sleep states, although one is diurnal and the other is more crepuscular.

Comment:
I have only minor concerns about some of the sleep deprivation methodology (see Additional comments below) and a bit more concern about the interpretation of the role of melatonin in the

two species. They conclude that melatonin is effective as a hypnotic in the jellyfish (which was also shown by Nath et al., 2017) but not in the anemone; however, the timing of the melatonin delivery may have missed the effective window—adding melatonin at ZT2-5 puts this at the sleep trough for jellyfish but at a relatively high sleep window for the anemone. In other words, sleep could be ceilinged out so melatonin can't boost sleep further, giving an artificial null result (adding melatonin to diurnal species like zebrafish also only really boosts sleep during the day and not at night when sleep and natural melatonin are already high). Testing melatonin later in the day, when sleep is lower, should be a straightforward way to bolster this claim.

Response:
We agree with the reviewer's comment and, as suggested, *treated both species with melatonin during their respective active (C. andromeda: ZT3; N. vectensis:* ZT13) and sleep periods (C. *andromeda:* ZT13; *N. vectensis:* ZT3). In both species, melatonin significantly promoted sleep during the active phase but had no effect during their natural sleep phase (Fig. 3j–u). We conclude that exogenous melatonin can promote sleep in both species, independent of their chronotype. Similar effects have been reported in other diurnal species (e.g., zebrafish[9]) and nocturnal animals (e.g., mice and worms[2,3]).

The new melatonin-related data are now described in the Results, Methods, and Discussion sections of the revised manuscript. Page 06, Line 187: *"We tested the effect of melatonin on sleep in the diurnal C. andromeda and crepuscular N. vectensis. While melatonin treatment did not affect sleep during the rest periods of N. vectensis (ZT3) and C. andromeda (ZT13) (Fig. 3j-l, s-u), sleep time, sleep bout duration, and the number of sleep bouts increased during the active phase in both N. vectensis (ZT13) and C. andromeda (ZT3) (Fig. 3m-o, p-r). These results suggest an evolutionarily conserved role of melatonin in sleep regulation across animals with different chronotypes"* Page 09, Line 298: *"…despite their opposing chronotypes, melatonin treatment promoted sleep in both species during their respective active phases, suggesting a conserved role for this hormone in sleep modulation."* Page 22, Line 646: *"Both species received either 100 μM melatonin (diluted in ethanol) or an ethanol control at ZT3 and ZT13, corresponding to their respective periods of wakefulness and activity."*

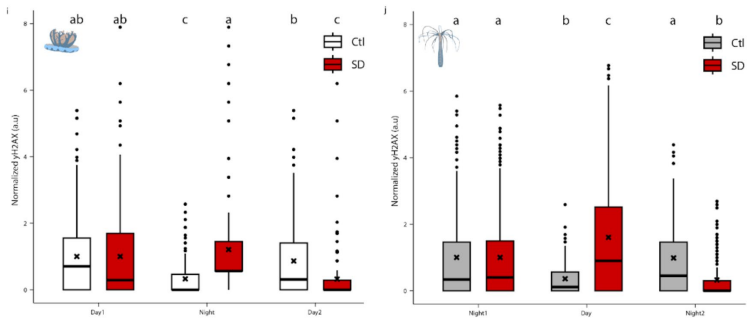


Comment:

The second half of the manuscript then examines the link between DNA damage and sleep, and here I find the analysis and interpretation of the results to be more muddled. Firstly, the independent experimental Ns for calculating DNA damage appears to be each cell (Figure 4g,h), but in how many independent animals? It isn't really appropriate to treat cells from the same animal as independent experimental replicates for statistical purposes; a more appropriate analysis would be average foci/cell calculated for each animal, and the N would be each animal. Thus, it is difficult from the data as presented to conclude if there are true differences in some of the conditions following sleep deprivation.

Response:

The analysis presented in Figure 4i–j focuses on a cellular phenotype rather than a whole-organism behavioral phenotype. Each neuron independently accumulates and repairs DNA damage and is therefore considered an individual experimental unit. Within a single animal, neuronal identity is highly variable and can be classified based on distinct genetic and activity profiles. In both *N. vectensis* and *C. andromeda*, the nervous system comprises diverse neuronal subtypes[13], which may respond differently to sleep and wake states. Indeed, we observed relatively high variability in neuronal DNA damage levels (Fig.4i, j). The analytical approach used in this study—immunostaining of nuclear γH2AX and treating individual neurons as independent experimental replicates—is well established in other model systems, including zebrafish[10,11], flies[14], and mice[14]. In response to this and other comments raised by the reviewer, we repeated the experiment, revised the sleep deprivation (SD) protocol, and are now presenting the new data in Figure 4i, j. Detailed statistical information and sample sizes are provided in the corresponding figure legend and in the methods section.



Comment:

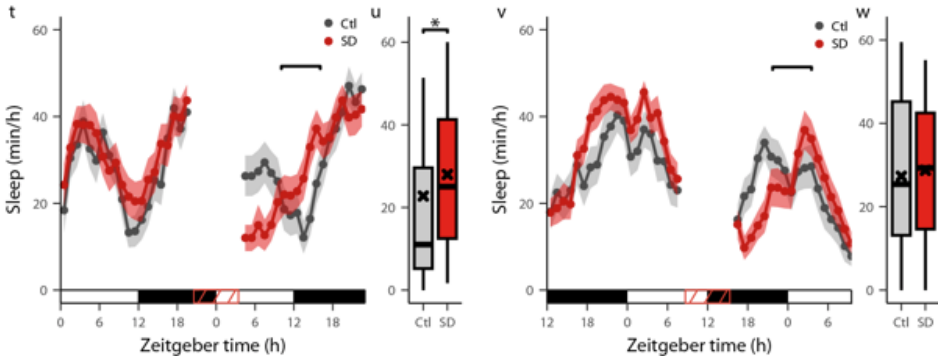
However, even if we take these results as described, some of the data appears to contradict the claim that sleep is important for resolving the DNA damage. For example, Figure 2t shows that a sleep deprivation from ZT0-6 leads to a substantial sleep rebound from ZT12-24. And yet, In figure 4h, the number of DNA damaged foci remains elevated at ZT17 following SD, even though these animals slept a lot more than the controls. This seems to contradict the conclusion of lines 180-183: “These experiments show that DNA damage increased during wakefulness and decreased during sleep in either diurnal or crepuscular cnidarians. Furthermore, these findings suggest that alleviating the levels of DNA damage, which accumulated during wakefulness, is an evolutionarily conserved cellular role of sleep”.

Response:

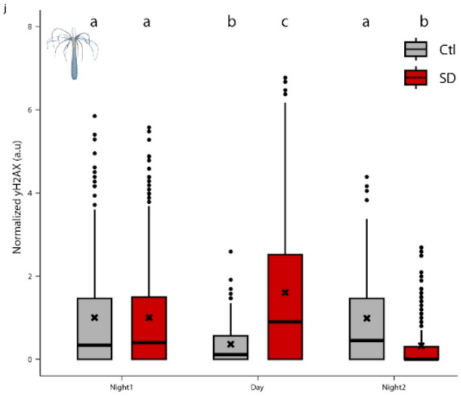
The link between DNA damage and sleep is supported by our findings in *C. andromeda*, which showed a reduction in DNA damage levels during the sleep rebound period (Fig. 4g, previous version). However, as noted by the reviewer, this trend was not observed in *N. vectensis*, where DNA damage levels did not decrease during the recovery period (Night 2, ZT17). Upon closer examination, we found that DNA damage did not significantly increase immediately after SD (ZT8) compared to baseline levels from the first day (ZT17), and no clear behavioral effect (i.e., reduced sleep immediately post-SD) was observed (Fig. 2t in the previous version). To address these limitations, we revised the sleep deprivation (SD) protocol. In the new version of the manuscript, animals were sleep-deprived by applying gentle, gradual water exchange once per hour during their sleep phase (ZT20-ZT4, marked by the red horizontal dashed line in Fig. 2t). This improved protocol effectively reduced sleep immediately post-SD (ZT 4), leading to a clear sleep rebound (Fig. 2t, u). In contrast, applying the same SD protocol during the peak of activity (ZT8–ZT15) did not result in a rebound (Fig. 2v, w), confirming that the effect is sleep-phase dependent. Importantly, under the revised protocol, DNA damage increased immediately following SD (ZT8), and significantly decreased during the rebound sleep phase at ZT17 of the second night (Fig. 4j). These results strengthen the conclusion that sleep facilitates the reduction of DNA damage.

In the revised version of the manuscript, the results are described in page 05, line 159*: “To test whether homeostatic process regulates sleep, we sleep-deprived the N. vectensis for 8h during sleep period (ZT20-ZT3) using gradual and gentle water exchange once per hour (Extended Data Movie 4). Following SD, sleep-deprived animals slept 1.24-fold more than control animals (Fig. 2t, u), indicating a homeostatic sleep drive. In contrast, control SD during the activity period*

(ZT8-ZT15) did not induce a sleep rebound (Fig. 2v, w).” The behavioral sleep results are presented in Fig. 2t-w:



In the revised version of the manuscript, the results are described in page 07, line 210*: “Under LD, the number of γH2AX foci increased at the end of the wakefulness period in C. andromeda (ZT11) and N. vectensis (ZT17) and reduced at the end of the sleep period in C. andromeda (ZT23) and N. vectensis (ZT8) (Fig. 4i, j)… Following SD, the number of γH2AX foci increased significantly in C. andromeda (ZT23) and N. vectensis (ZT8) and subsequently decreased following sleep recovery (Fig. 1o, p; Fig. 2t, u) in both species (Fig. 4i, j)”* The effect of SD on DNA damage is presented in Fig. 4j:



The SD protocol is explained in the methods section page 22, line 634: *“In SD experiments, the animals were stimulated by gentle water exchange once per hour during their peak of sleep period (ZT20-ZT3) and peak of activity period (ZT8-ZT15; Extended Data Movie 4) and sleep was monitored before and after the SD.”*

Comment:

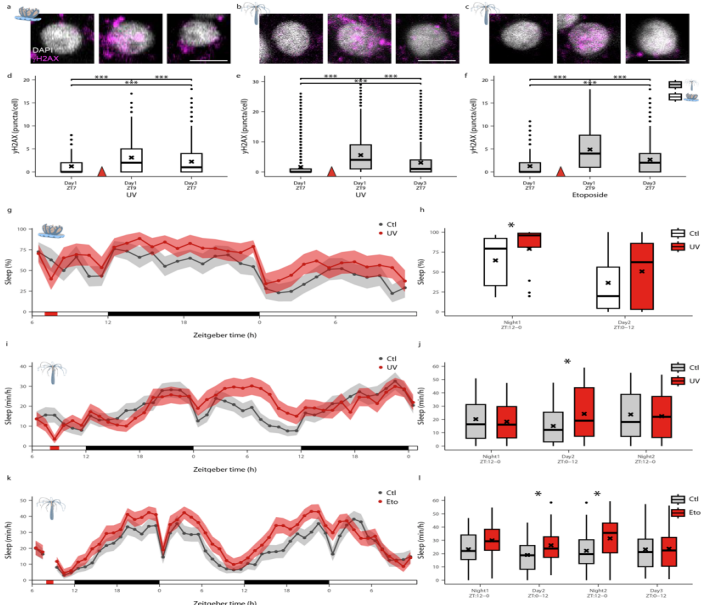
Finally, the authors use UV light to induce DNA damage and claim that sleep is increased, thus showing a bi-directional relationship between DNA damage and sleep. However, the data are shown only as box-whisker summary plots, making them difficult to evaluate in terms of quality of the results. Better would be to display these as timecourse data, before and after UV exposure, and for a longer window than the summary—the post UV sleep is reaching 100% for jellyfish and 60/60 minutes for the anemone, so more convincing evidence that these animals are not permanently harmed by the UV exposure would be critical here. Even better would be to show

that the DNA damage eventually resolves (by the next day or something) and that the sleep recovers as well.

Response:
We thank the reviewer for this important conceptual comment. As requested, we conducted a ~2-day time-course monitoring of sleep behavior and DNA damage before and after UV exposure in both *C. andromeda* and *N. vectensis*. UV irradiation was applied at ZT8 (indicated by a red triangle in Fig. 5X–X or a red line in Fig. 5X–X), and it promoted increased sleep, which occurred during the subsequent night in *C. andromeda* and during the day in *N. vectensis*, consistent with their respective chronotypes. In parallel, UV exposure led to a significant increase in DNA damage at ZT9. Following the sleep recovery period, DNA damage levels returned to baseline (Fig. 5X–X). The normalization of DNA damage suggests that the animals were not permanently affected by the relatively short UV exposure. These findings support the conclusion that UV-induced DNA damage promotes sleep, and that sleep, in turn, facilitates the repair and reduction of DNA damage.

We describe these findings in the Results section (Page 08, Line 229): *"In C. andromeda and N. vectensis, the number of γH2AX foci increased one hour after UV exposure (Fig. 5a, b, d, e). Subsequently, nighttime and daytime sleep increased in C. andromeda (Fig. 5g, h) and N. vectensis (Fig. 5i, j), respectively. Notably, following sleep recovery, the animals remained viable, and two days later the levels of DNA damage reduced in both species (Fig. 5a, b, d, e). These results suggest that UV-mediated induction of DNA damage increases sleep pressure… These results show that elevated DNA damage can promote sleep in early divergent lineage. Moreover, these inter-species comparative findings suggest that environmental factors, such as UV radiation and soluble mutagenic compounds, which can induce DNA damage and cellular stress, may have contributed to the evolutionary emergence of sleep in cnidarians."*

These new results are presented in Fig. 5a–j.



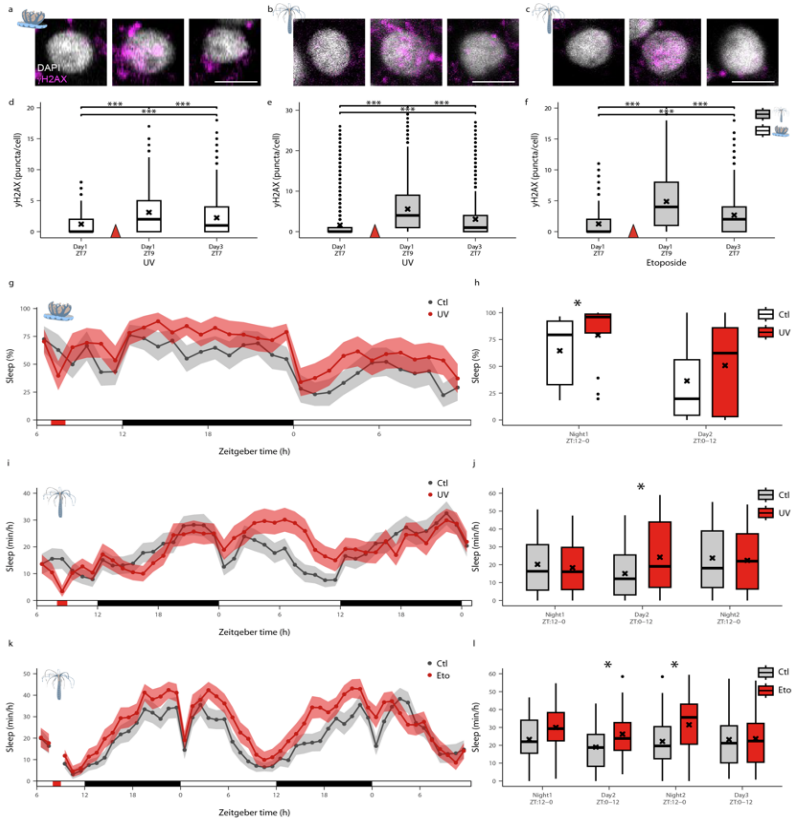
Comment:
To be more convincing, it would also be good to show that DNA damage induced in another manner also increases sleep—for example these creatures have a host of natural behavioral responses to UV light (see for example Lilly et al., 2024, where the larval anemones shift their behavior to a planktonic swimming in direct response to UV), so showing the sleep changes are due to the damage and not UV-activated neural pathways would go far to convince.

Response:

The results presented in Fig. 5a–j suggest that UV-induced DNA damage promotes sleep. As suggested by the reviewer, and to further support this finding, we induced DNA damage using an alternative method. Animals were treated with etoposide, a well-established mutagenic compound. While UV exposure induces oxidative stress and DNA base damage, etoposide causes DNA damage by inhibiting topoisomerase II, thereby preventing effective DNA repair.

Etoposide treatment led to increased DNA damage, followed by an increase in sleep. Notably, DNA damage levels decreased after the recovery sleep period the following day (Fig. 5c, f, k, l). This experiment provides an independent, light-free method to induce DNA damage and further supports the conclusion that DNA damage promotes sleep (Fig. 5k, l).

These results are described in the Results section (Page 08, Line 234): *Since UVB may also affect the behavior of cnidarians in alternative pathways[46], we treated N. vectensis with the mutagenic compound Etoposide[47]. While UVB induces oxidative stress and damage to DNA bases, Etoposide inhibits the topoisomerase II enzyme and stabilizes DNA double strand break formation. Similar to the effect of UV, Etoposide treatment abruptly increased the number of γH2AX foci (Fig. 5c, f). The animals then increased sleep during the following day and night (Fig. 5k, l), and DNA damaged was reduced (Fig. 5c, f). These results show that elevated DNA damage can promote sleep in early divergent lineage.".* The results of the etoposide treatment are presented in Fig. 5c, f, k, l.



Comment:
To conclude, I am convinced that anemones (and jellyfish) have a sleep state, and this alone is a nice contribution. However, I am less convinced that the DNA-damage sleep hypothesis has been so clearly demonstrated in these animals based on the current data presented.

Response:
We believe that our detailed responses, corresponding text modifications, and the additional experiments—namely: (1) the effect of the revised sleep deprivation protocol on sleep and DNA damage; (2) melatonin treatments administered during both day and night; and (3) continuous quantification of DNA damage and sleep following UV and etoposide treatments—collectively strengthen the manuscript. These new data provide compelling evidence for a bi-directional relationship between DNA damage and sleep in both the diurnal *C. andromeda* and the crepuscular *N. vectensis.*

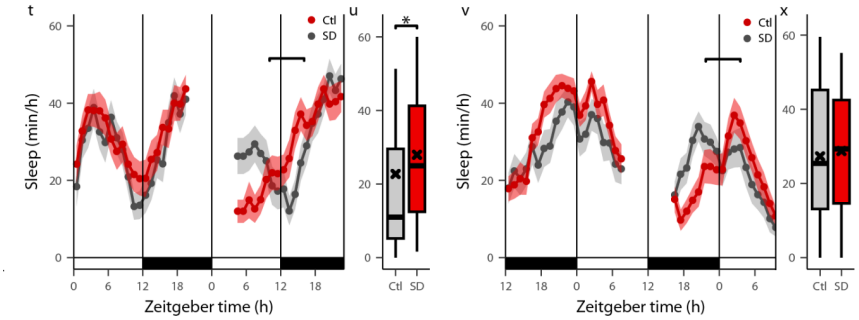
Additional and minor comments:

Comment:
Figure 2—The arousal threshold data, circadian and clock mutant data are all very good. The Sleep deprivation data is good but a few additional controls would be useful. For example, the rebound is shown after depriving the animals of the daytime siesta, but the sleep traces show a lot of sleep in the latter half of the night as well—even more in fact. Showing SD for this window would also be good, as in other species like Drosophila is not totally clear that the siesta and night

sleep are identically regulated. Perhaps more important is to use the SD method at a time of relative wakefulness and control for rebound due to physical exhaustion of being kept awake, a standard test in the field.

Response:
As recommended by the reviewer, we revised the sleep deprivation (SD) method and applied it during both the sleep period (ZT20–ZT4) and a period of relative wakefulness (ZT8–ZT16). A clear sleep rebound was observed only following SD administered during the sleep period, indicating that sleep loss must occur during the natural rest phase to elicit a homeostatic response.

These findings are described in the Results section (Page 05, Line 159): *"To test whether homeostatic process regulates sleep, we sleep-deprived the N. vectensis for 8h during sleep period (ZT20-ZT4) using gradual and gentle water exchange once per hour (Extended Data Movie 4). Following SD, sleep-deprived animals slept 1.24-fold more than control animals (Fig. 2t, u), indicating a homeostatic sleep drive. In contrast, control SD during the activity period (ZT8-ZT16) did not induce a sleep rebound (Fig. 2v, w)."* The SD protocol is described in the methods section (page 22, line 634): "In SD experiments, the animals were stimulated by gentle water exchange once per hour during their peak of sleep period (ZT20-ZT4) and peak of activity period (ZT8-ZT16; Extended Data Movie 4) and sleep was monitored before and after the SD." The SD data is presented in Fig. 2t-w



Comment:
Figure 2—the SD method uses light, which may be altering circadian clock function and timing as well as sleep deprivation. Either a non-light method for SD should be used, or show that clocks aren't shifted by the light pulses.

Response:
As requested by the reviewer, and to minimize potential effects on the circadian clock, we employed a non-light-based method for sleep deprivation—specifically, gradual and gentle water exchange.

Comment:
Figure 2- methods; the details for the light stimulation SD are not provided in the methods as far as I can see, including light levels, etc.

Response:
In the revised manuscript, we no longer use light-based stimulation for sleep deprivation. Instead, we implemented a protocol involving gentle, gradual water exchange once per hour. This method is described in the Methods section and demonstrated in Extended Data Movie 4.

The following text has been added to the Methods section page 22, line 634: *"In SD experiments, the animals were stimulated by gentle water exchange once per hour during their peak of sleep period (ZT20-ZT3) and peak of activity period (ZT8-ZT15; Extended Data Movie 4) and sleep was monitored before and after the SD."*

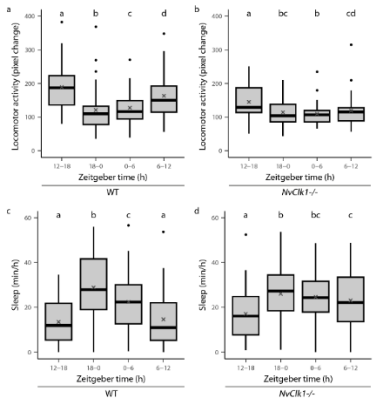
Comment:
Figure 2t—if light is being used to sleep deprived, it isn't clear to me why there is a gap in the data—it would be better to track the animals during the SD to see exactly how much sleep is lost. I can understand why there is a gap in Figure 1, where the SD is done physically that will interfere with the tracking, but light should be straightforward to track.

Response:
Thank you for the feedback. During sleep deprivation, whether using the previous light-based protocol or the revised method involving gentle hourly water exchange, animals were removed from the imaging setup, making it impossible to continuously track their sleep behavior during the SD period (Extended Data Movie 4).

Comment:
Figure 2m—defining the windows of analysis across the dark-light transition (ZT18-6 and 6-18) is a bit odd, I would just break it down into night 2nd half and day first half to be a little clearer.

Response:
We appreciate the reviewer's suggestion and agree that the ZT18-6 and 6-18 is rather unconventional. To maintain consistency with the 12-hour time windows commonly used under light or dark conditions (e.g., Fig. 1l), we identified the sleep and wake periods of *N. vectensis* under LD conditions (Fig. 2l, Fig. 4b) and defined the 12-hour analysis windows accordingly. Although we acknowledge that this division (ZT18–6 for sleep, ZT6–18 for wake) is unconventional, it was necessary given the crepuscular nature of the animals and permits direct cross-species comparison.

Following the reviewer's suggestion, we expanded the explanation for this choice in the results page 4, line 121: *"We analyzed 6h bins (Extended Data Fig. 4) but chose 12h windows (ZT18-6 and ZT6-18) because they encompass the full crepuscular (i.e., active at dusk) behavioral pattern and align with the 12h light/dark periods used for C. andromeda (Fig. 1m), facilitating cross-species statistics.".* The data is presented Extended data Fig.4:



Comment:
Figure 3J—100 mM melatonin must be a typo, and it is listed as 100uM in the methods (and that could still be considered high). To convince that melatonin plays no role in the sleep of the anemone, adding melatonin at different timepoints will be critical (see main comments).

Response:
Good catch—thank you. This typo error was corrected. We used 100 µM melatonin, consistent with concentrations previously used in *C. xamanacha*[15]. As explained above and in line with the reviewer's recommendation, we treated the animals with melatonin at different time points to assess its effects during both their active and sleep phases.

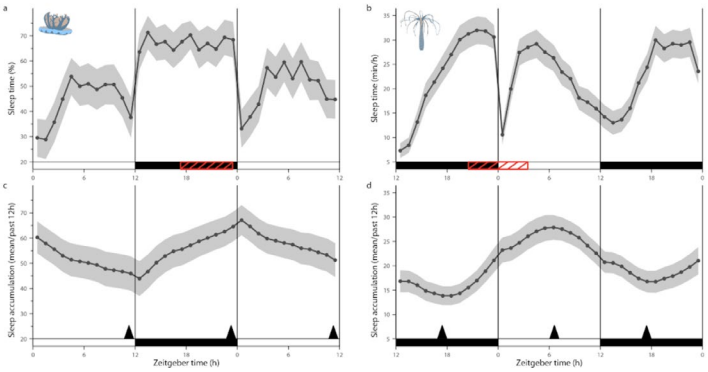
Comment:
The term "sleep quality" is too loaded a term—it implies something about a "good" night or a "bad" night of sleep but it is impossible to say that fragmented sleep or consolidated sleep is of more or lesser quality without showing effects on performance the next day. I would recommend "sleep structure" or "sleep architecture" or similar when looking at sleep bout counts and lengths.

Response:
As recommended by the reviewer, we replaced the term "sleep quality" with "sleep architecture" throughout the manuscript.

Comment:
Figure 4: The SD experiment for jellyfish is from ZT17-23, but for *N. vectensis* is ZT0-8. ZT0-8 does not quite line up with maximal sleep in this species ZT18-24 or ZT18-6 makes better alignment. Figure 4A, showing the rolling 12 hour previous sleep time is misleading on this – the figure 4b data is high already at ZT0 and peaking at ZT6, but that means the sleep is maximal in the PREVIOUS 12 hours, which is when the SD should be done for testing the effect of SD on sleep. Plotting it this way is visually confusing compared to Figure 2L, where the sleep timing is easier to appreciate.

Response:
In accordance with the reviewer's suggestion, we sleep-deprived *N. vectensis* during its peak sleep period (ZT20–ZT3), which resulted in a clear sleep rebound. To help readers better

understand the rationale behind the selected SD time window, we plotted the sleep time data above the sleep accumulation data in Fig. 4. The rationale is explained in the results section page 07, line 200*: "We hypothesized that DNA damage would peak and decline following extended wake and sleep, respectively. To identify the time points of maximal and minimal sleep pressure, we calculated the average sleep time over the past 12h that preceded each time point."* The sleep deprivation period is indicated by a red dashed line in Fig.4a and b:



Comment:
Figure 4: If sleep is really important for repairing the DNA damage, then why does it not recover in the anaemone after SD from ZT0-8? It is confusing because they sleep more from 12-17 then they even do during the "SD" ZT0-8 window, so if this were all about sleep driving the recovery, then wouldn't we expect it to be recovered? Figure 3 shows after SD, the anemones sleep more, so this should have resolved the foci if the interpretation were correct. This seems counter to the conclusion of line 180-183: "These experiments show that DNA damage increased during wakefulness and decreased during sleep in either diurnal or crepuscular cnidarians. Furthermore, these findings suggest that alleviating the levels of DNA damage, which accumulated during wakefulness, is an evolutionarily conserved cellular role of sleep.

Response:
As addressed above, a revised sleep deprivation (SD) protocol was applied during the sleep period (ZT20–ZT3). This protocol led to an increase in DNA damage and induced a sleep rebound, which was subsequently followed by a reduction in DNA damage levels.

Comment:
Figure 4: The data is not showing independent Ns…. The legend says n=291 cells, but how many animals? One cannot use a cell in the same animal as an independent measure for the stats unless you control for them as dependent on each other. What stats are being done in Figure 4? The legend doesn't say. It says Tukey, but that is a post-hoc test. It also lists a lot of cross comparisons; how is this being controlled for multiple comparisons? There are really two-factors, SD/no SD and time, so a 2-factor analysis design would be clearer.

Response:
The analysis presented in Figure 4i–j focuses on a cellular phenotype rather than a behavioral phenotype. As explained above, individual cells are treated as the experimental units in this

analysis. As requested by the reviewer we added statistical details and sample sizes in the corresponding figure legend and in the methods section. The revised legend now specifies the full statistical workflow of two-way ANOVA (treatment × time) followed by Tukey HSD, corrected for multiple comparisons.

Comment:
Figure 5: the post-UV sleep data is too summarized. IT would be better to see the time course of the behavior afterwards. Also, the recovery on the following day—sleep is being driven so high after UV are you sure they aren't permanently harmed, i.e. sleeping a lot but actually damaged?

Response:
As addressed above, and in response to the reviewer's suggestion, we performed a time-course analysis of behavior before and after UV irradiation. In parallel, we quantified DNA damage at three time points: before UV exposure, immediately after irradiation, and following the recovery sleep period. The animals remained viable and fully recovered from the UV treatment. These results are presented in Fig. 5.

Comment:
5e and 5f are not presented in the same manner, one show ZT8-12 and ZT12-0, the other says Pre: Post, but over what windows constitute "pre" and what consistutes "post"? The legend says 1hr pre and 1 hr post, but that seems like too short a time? What would be better would be to induce UV damage, see how long the damage takes to be resolved and correlate that timecourse with the sleep timecourses— i.e. once the damage is resolved, does sleep end?

Response:
We have corrected the terminology and now refer to the sampling time points using ZT values. These updates are reflected in Fig. d–f.

Comment:
Another method to induce DNA damage? These species are quite sensitive behaviorally to UV light directly, for example the anemone in the larval state are induced by UV into plaktonic swimming (Lilly et al 2024). Can you distinguish between these two models UV◊DNA damage◊ sleep vs. UV◊ sleep (independent of the DNA damage?

Response:
Yes, we used an additional method to induce DNA damage. As explained above, and to support the role of UV-mediated DNA damage specifically—rather than alternative pathways—we treated animals with etoposide, a well-established inducer of DNA damage via topoisomerase II inhibition. Etoposide treatment also led to increased sleep, consistent with a DNA damage-mediated sleep response. Notably, in both the UV and etoposide treatments, DNA damage levels decreased following the sleep rebound period (Fig. 5), further strengthening our conclusion that sleep facilitates the reduction of DNA damage.

Nonetheless, we do not exclude the involvement of additional mechanisms. This point is acknowledged in the Results section. These results are described in the Results section (Page

08, Line 234: "*These results suggest that UV-mediated induction of DNA damage increases sleep pressure. Since UVB may also affect the behavior of cnidarians in alternative pathways[46], we treated N. vectensis with the mutagenic compound Etoposide[47]. While UVB induces oxidative stress and damage to DNA bases, Etoposide inhibits the topoisomerase II enzyme and stabilizes DNA double strand break formation. Similar to the effect of UV, Etoposide treatment abruptly increased the number of γH2AX foci (Fig. 5c, f). The animals then increased sleep during the following day and night (Fig. 5k, l), and DNA damaged was reduced (Fig. 5c, f). These results show that elevated DNA damage can promote sleep in early divergent lineage."* The results of the etoposide treatment are presented in Fig. 5c, f, k, l. And we cite Lilly et al., 2024 to support this broader context.

Comment:
The evolutionary claim seems too strong: "Altogether, these inter-species comparative findings suggest that environmental stressors, such as UV radiation, which can induce DNA damage and cellular stress, drove the evolutionary origin of sleep in the day or crepuscular active cnidarians." What exactly is the evidence that this was the "driver" of evolution? Showing that DNA damage induces sleep and sleep resolves DNA damage in these species isn't quite the same as saying that this relationship is what "drives" evolution of sleep.

Response:
We have moderated this hypothesis in the revised manuscript. The updated text now reads: (page 11, line 350*): "Moreover, considering that the interplay between the circadian clock and genomic stability has been shown in non-neuronal mammalian cultured cells, fungi and cyanobacteria[78–81], sleep-like states may have preceded the evolution of neurons, originally evolving to support synchronized and consolidate periods of cellular maintenance."*

References

1. Mascetti, G. G. Unihemispheric sleep and asymmetrical sleep: behavioral, neurophysiological, and functional perspectives. *Nat Sci Sleep* **8**, 221–238 (2016).

2. Kim, P. *et al.* Melatonin's role in the timing of sleep onset is conserved in nocturnal mice. *NPJ Biol Timing Sleep* **1**, 13 (2024).

3. Omond, S. *et al.* Inactivity Is Nycthemeral, Endogenously Generated, Homeostatically Regulated, and Melatonin Modulated in a Free-Living Platyhelminth Flatworm. *Sleep* **40**, (2017).

4. Pregueiro, A. M., Liu, Q., Baker, C. L., Dunlap, J. C. & Loros, J. J. The Neurospora Checkpoint Kinase 2: A Regulatory Link Between the Circadian and Cell Cycles. *Science* **313**, 644–649 (2006).

5. Liao, Y. & Rust, M. J. The circadian clock ensures successful DNA replication in cyanobacteria. *Proceedings of the National Academy of Sciences of the United States of America* **118**, (2021).

6. Gamsby, J. J., Loros, J. J. & Dunlap, J. C. A phylogenetically conserved DNA damage response resets the circadian clock. *J Biol Rhythms* **24**, 193–202 (2009).

7. Oklejewicz, M. *et al.* Phase Resetting of the Mammalian Circadian Clock by DNA Damage. *Current Biology* **18**, 286–291 (2008).

8. Durieux, D. M. *et al.* Benthic jellyfish act as suction pumps to facilitate release of interstitial porewater. *Sci Rep* **13**, 3770 (2023).

9. Appelbaum, L. *et al.* Sleep-wake regulation and hypocretin-melatonin interaction in zebrafish. *Proc Natl Acad Sci U S A* **106**, 21942–21947 (2009).

10. Zada, D., Bronshtein, I., Lerer-Goldshtein, T., Garini, Y. & Appelbaum, L. Sleep increases chromosome dynamics to enable reduction of accumulating DNA damage in single neurons. *Nat Commun* **10**, 895 (2019).

11. Zada, D. *et al.* Parp1 promotes sleep, which enhances DNA repair in neurons. *Mol Cell* **81**, 4979-4993.e7 (2021).

12. Noubissi, F. K. *et al.* Detection and quantification of γ-H2AX using a dissociation enhanced lanthanide fluorescence immunoassay. *Sci Rep* **11**, 8945 (2021).

13. Sprecher, S. G. Neural Cell Type Diversity in Cnidaria. *Front Neurosci* **16**, 909400 (2022).

14. Bellesi, M., Bushey, D., Chini, M., Tononi, G. & Cirelli, C. Contribution of sleep to the repair of neuronal DNA double-strand breaks: evidence from flies and mice. *Sci Rep* **6**, 36804 (2016).

15. Nath, R. D. *et al.* The Jellyfish Cassiopea Exhibits a Sleep-like State. *Curr Biol* **27**, 2984-2990.e3 (2017).

Reviewer #1 (Remarks to the Author):

The authors have effectively addressed my concerns. I have nothing new to add.

Response:
We thank you very much for supporting our paper.

Reviewer #2 (Remarks to the Author):

The revised manuscript has addressed many of the concerns raised in the previous round, and I acknowledge a clear overall improvement in both clarity and scientific rigor. In particular, the experimental validation of sleep phenomena in C. andromeda and N. vectensis appears robust and convincing. The study adds valuable insights into sleep regulation in cnidarians and constitutes a meaningful contribution to the field.

Response:
We appreciate your kind words and your support for our manuscript.

Comment:

However, regarding the central claim expressed in the title—namely, that "DNA damage is a sleep driver in ancient cnidarians"—I believe further caution and refinement are required.

Response:
As suggested by the reviewer, we conducted additional experiments (detailed below) that further support our central claim. In addition, we refined the title, which now reads: "*DNA damage modulates sleep drive in basal cnidarians with divergent chronotypes*."

Comment:

Specifically, this claim demands greater precision and strength in both experimental design and data analysis. I outline below several key issues that I recommend the authors address in the current revision.

1. Y-axis scaling and quantification in Figure 4i,j.

The vertical axis in Figure 4i,j lacks sufficient explanation, making it difficult to interpret. If the same quantification method (i.e., puncta/cell) as in Figure 5d–f was used, then I suggest that the graphical representation should be standardized across figures for consistency.

Conversely, if a different quantification method was employed, then the authors should explain why the approach used in Figure 5 was not applied here. In that case, the rationale for using an alternative method should be clearly justified from a scientific standpoint. Overall, the quantification approach should be unified where possible.

Response:
A unified quantification method (puncta per cell) was applied in both Figures 4 and 5. Following the reviewer's suggestion, we standardized the Y-axis labeling across figures to "Normalized number of γH2AX foci (a.u.)." The updated charts are now

presented in Figures 4 and 5, and the corresponding legends have been revised accordingly.

Comment:

2. Statistical annotation (Figure 4i,j and other figures).

The notation "different lowercase letters above the bars indicate statistical significance at $p < 0.05$, Tukey HSD, two-tailed; FWER controlled" is vague and difficult to interpret. I strongly recommend that the authors explicitly state which groups were compared and the nature of the statistically significant differences. Similar statistical annotations appear in other figures, including supplementary figures. A more intuitive and consistent presentation would enhance interpretability across the manuscript.

Response:
As recommended by the reviewer, we clarified the statistical representation in the revised manuscript. Rather than applying Tukey HSD to all pairwise group combinations, we now use Mann–Whitney tests to compare only the groups of interest. This approach simplifies the result presentation, directly addresses the experimental question, and aligns the analysis with the methods used elsewhere in the paper. Importantly, the revised statistical analysis did not alter the conclusions. Furthermore, the updated approach highlights the specific comparisons of interest, and the nature of statistically significant differences is explicitly stated in all figure legends. These changes have been implemented across both the main and supplementary figures.

Comment:

3. Relationship between sleep pressure and DNA damage.

The current manuscript presents compelling evidence that DNA damage accumulates during wakefulness and is reduced during sleep under light–dark conditions. It also demonstrates that short-term sleep deprivation (SD) induces DNA damage, supporting the hypothesis that sleep contributes to DNA repair. These are important findings.

Response:
Thank you for the supportive comment and the summary of one of the key conclusions of the manuscript.

Comment:
However, to convincingly support the central claim that "DNA damage is a sleep driver," I believe further experimentation is needed. Specifically, I encourage the authors to explore whether prolonged sleep deprivation-e.g., over 24 hours or multiple days-leads to a measurable accumulation of DNA damage. This would provide critical support for the proposed causal relationship between sleep pressure and DNA damage.

Response:
We calibrated the timing and duration of sleep deprivation (SD) and determined that 6 hours (ZT17–ZT23, Fig. 1o) and 8 hours (ZT20–ZT4, Fig. 2t) represent optimal SD periods. These conditions consistently caused a robust homeostatic sleep response (Fig. 1o, p; Fig. 2t, u) and elevated DNA damage levels (Fig. 4k, l). Our empirical tests

showed that extending SD beyond 24 hours increased the likelihood of non-sleep-specific stress responses. In both *Cassiopea* and *Nematostella*, prolonged SD led to tissue degradation and disintegration. Similar confounding effects of extended SD have been documented in other animal models (Vaccaro et al., 2020; Sang et al., 2023; Wu et al., 2023), complicating the interpretation of sleep-specific mechanisms. Accordingly, we adopted an SD protocol optimized for both efficacy and animal viability, providing a physiologically relevant framework for investigating the role of sleep in regulating DNA damage.

Comment:

Furthermore, the authors have shown that melatonin induces sleep during the active phase but has no effect on sleep duration during the sleep phase. This feature could be leveraged experimentally. For example:

Does melatonin-induced sleep during the active phase reduce DNA damage? Does melatonin administration during the sleep phase alter DNA damage independently of sleep induction?

These complementary experiments would allow the authors to distinguish between the antioxidative effects of melatonin and the role of sleep per se in promoting genomic stability. I believe that these additions would significantly strengthen the manuscript and make the central claim more persuasive.

Response:
We agree with the reviewer that testing the effect of melatonin-induced sleep could strengthen one of the manuscript's conclusions. Following this recommendation, we examined the effect of melatonin on DNA damage during the active period in both *C. andromeda* (ZT13; Fig. 5d, i) and *N. vectensis* (ZT3; Fig. 5e, j). Melatonin treatment reduced DNA damage levels in both species, consistent with its sleep-promoting effect (Fig. 3m, p). These results further support the conclusion that sleep alleviates DNA damage in both cnidarians. The additional melatonin experiments are presented in Fig. 5d, e, i, j and are described in the Results and Methods sections of the revised manuscript. Potential additional effects of melatonin as an antioxidant are discussed in the Discussion section (starting at line 301).

Comment:
4. Number of animals used in DNA damage assays.

While the number of cells analyzed appears sufficient, the number of animals examined seems relatively low. Given that sleep-related behavioral metrics (e.g., bout duration, total sleep time) were calculated from a large number of animals, the DNA damage experiments should ideally match that level of biological replication. To ensure comparability and statistical robustness, I recommend increasing the number of animals used in the DNA damage assays to align with those used in the sleep behavior analyses.

Response:

As recommended by the reviewer, in the revised version of the manuscript, we repeated all DNA damage quantification experiments and increased the animal sample size to match the level of biological replication used in the behavioral assays. While the additional data improved statistical robustness, it did not change the conclusions of the previous version. The new data sets are incorporated into Figs. 4 and 5, with the number of animals indicated in the corresponding figure legends. The Results and Methods sections have been revised accordingly.

Reviewer #3 (Remarks to the Author):

The authors have mostly addressed the reviewers' comments and my concerns, including with important new experiments that greatly improve the manuscript.

Response:

We thank the reviewer for the kind and supportive comments.

Comment:

With one very important exception that must be resolved before I could consider acceptance of this paper.

In my original review, I raised an issue of statistical pseudoreplication that comes from treating neurons within the same animal as "independent". Their response was not to correct this but instead claim that they "focus on a cellular phenotype" and that "each neuron independently accumulates and repairs DNA damage and is therefore an individual experimental unit." I very strongly disagree with this claim, on scientific first principles. In support, the authors state this is an established method by citing their own papers! Even if this is something that was done in other papers, it doesn't make the use of this approach in this paper right; it just means that those other papers are also doing this analysis incorrectly.

A few simple thought experiments can illustrate the point that neurons in the same animal are not, and cannot, be treated as statistically independent. First, consider an experiment where you expose some hydra to UV to see if DNA damage is resolved by sleep. At the first timepoint post UV, you grab a single animal that happened to be closer to the UV source for the duration of the experiment, and then you count DNA damage foci in 100s of neurons in that single animal. Now, on the second timepoint after sleep, you grab a single hydra that happened to stay far from the UV source (darn chance!), and count 100s of neurons. Is the n=100s for each group, or is the n=1? If you use n=100, even a very tiny effect, possibly caused by differences in UV exposure alone, will have some very small p-value, and you will rejoice that your experiment "worked". But if you use n=1, you will correctly surmise that the n is too small to draw a meaningful conclusion and you won't be misled by a chance result.

Even though the idea that n=100 can come from one animal already makes the point pretty obvious, maybe one can try to argue that all the animals must get the same UV. Consider a second thought experiment then, in which a single neuron in an animal gets zapped by UV that generates some DNA damage that causes it to become

hyperactive. This single neuron then causes downstream neurons to become active as well, which we know can trigger DNA damage in those cells. You see? The DNA damage accumulating in neurons doesn't happen independently of the other neurons in that animal, so they cannot be treated as independent. To do so inflates artificially the n, which for statistical purposes must be independent of each other. This is why in Figure 4 and 5, the authors report such small p-values, despite a fairly variable set of data—the n is inflated.

This fundamental misunderstanding does make me worried about how other analyses were done in the paper, but I don't see anything clearly wrong in other figures.

Response:

We appreciate the reviewer's concern and agree that the animal is an ideal experimental unit (n). Nonetheless, in molecular neuroscience, particularly neurophysiology, it is standard to consider individual cells as experimental units, especially when studying DNA damage, neuronal activity, and other cell-specific processes. Neurons are highly heterogeneous in both activity and genetic identity (e.g., excitatory glutamatergic vs. inhibitory GABAergic) and are therefore frequently treated as independent units. The reviewer suggested that "DNA damage accumulating in neurons doesn't happen independently of the other neurons." However, neuronal responses are heterogeneous within a single animal: some cells are excitatory, others inhibitory, and their activity is differentially modulated. Consequently, DNA damage does not accumulate uniformly; it may increase in active cells while remaining lower in inhibited ones. Moreover, neuronal activity is only one of several contributing factors (e.g., ROS, enzymatic errors, chromatin dynamics, radiation). Our data, consistent with the broader literature, support that DNA damage accumulation is cell-specific. If neurons responded uniformly, homogeneous levels would be expected across cells, which is not observed.

However, in response to the reviewer's recommendation, we repeated all experiments shown in Figs. 4 and 5, increased the number of animals analyzed, and re-analyzed the data statistically. In *N. vectensis*, the statistical unit is now animals (n=animals). In the larger *C. andromeda*, which has a long life cycle and challenging reproduction, the statistical unit is the ganglion, a morphologically and functionally distinct pacemaker unit with independent activity patterns (Lyndby et al., 2023, PMID: 37752841; Abrams et al., 2025, PMID: 40658847). In both species, DNA damage was quantified across dozens of cells per unit. The numbers of ganglia and animals are explicitly reported in the figure legends. As anticipated, the additional data and revised analysis did not alter the conclusions of the original manuscript.

Finally, to further strengthen our findings, we conducted additional experiments showing that melatonin treatment during the active period promoted sleep (Fig. 3m–o, p–r) and reduced DNA damage levels (Fig. 5d, i; e, j) in both species.