

The cost of re-synchronization in a leaf-cutter ant colony

Corresponding Author: Ms Mila Maria Pamplona Barbosa

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors examined the daily activity rhythms of leaf-cutting and foraging in the leaf-cutting ant and their re-entrainment to a shifted light-dark cycle using a video recording system. They found significant rhythmicity in those activities and changes in those activities during resynchronization. The results seem to have potential interests. Unfortunately, however, description of the methods and results seems poor. The authors missed important points to describe in the Methods and Materials and Results sections. Discussion is also poor and some includes discrepancy with the results. The sentences or paragraphs are often incomplete, being broken in the middle. The authors should be careful to write their descriptions without omissions and to ensure that their arguments are coherent. I also offer the following specific comments for the authors' consideration.

1. The recording for daily activity rhythms was intermittent, taking place every 20 min for 2 min. So data are not available most of time. Therefore, the authors should discuss the validity of their recording and analysis methods.
2. Regarding the recordings, they state that the record was performed for 120 min before and after leaf replenishments when analyze the masking effects of leaf replenishments. However, they do not state how they analyzed the data. They should explain the method appropriately. For example, what was the time span unit for the analysis?
3. L118: The materials of the colony maze should be mentioned. Is this made of transparent plastic? Can light penetrate into the maze? Are all ants exposed to light during the light phase? These are very important information for the entrainment experiment.
4. L233: Please explain what is AUC? How did you calculate AUC?
5. L270: influenced ant rhythms
6. L280: Here you should show actual value of AUCs then you should show the result of statistical tests. You should clearly describe which values were statistical compared.
7. L306: This sentence is uncompleted. There is no description on data shown in Figure 4.
8. Figure 3A: Please add label of abscissa. Ordinate labels should be Foraging and Leaf cutting rate.
9. Figure 3BC: Time of day on the top of each panel should be deleted. Instead the time of leaf replenishment is better. Please move label of abscissa to an appropriate place.
10. Figure 3D: average daily rhythms: How many days did you use for the average? Especially for Stage II, what are the days? Why do you include Stage II data here? Ordinate labels should be provided.
11. Line 324: "As of the 6th day of stage II (days 16 to 20)": This is very confusing.
12. Line 324: "we recovered the pattern described for stage I" Please consider to say the pattern observed in Stage I was restored.
13. Line 326: This sentence is incomplete.
14. L314: The authors examined the correlations between leaf cutting rates and ant flow and compared them between stages. However, no actual values and significance were stated. These should be clearly mentioned.
15. L328: Please consider to say "Daily changes in the ratio between...."
16. L342: $P=0.07$ shows not significant.
17. L348: daily leaf intake in the dark phase during the first 5 days
18. L349: after day 15.
19. L354: insert period after II.
20. L368: Why could you say the colony is particularly sensitive near 0h? In Fig. 3B, there is no strong evidence for this statement. There is no statement in the Results section regarding this point. The authors should describe important points of the effects of leaf replenishment in the results section.
21. L327: "Our results indicated..." I don't understand the meaning of this sentence.
22. L372-282: In this paragraph, the authors discussed the re-entrainment to the delayed LD and concluded the re-entrainment does not complete within 10 days after the shift. However, in other places, they discussed their results assuming that the re-entrainment is accomplished during the first 5 days of the shift. When look at Figure 3D, the daily distribution of activity is quite similar between Stage I and II, suggesting complete re-entrainment of the rhythms. So the discussion is quite confusing to this reviewer. Resynchronization and criteria for determining re-entrainment needs to be properly defined.

Reviewer #2

(Remarks to the Author)

Major comments:

1. The Introduction needs a section speaking about the basic biology of *Atta sexdens*. This would help readers who are not familiar with *Atta sexdens* and their life history. For example, the importance of fungi for these ants was first mentioned in the discussion section.
2. While the statistical methods mentioned in the methods section are appropriate, the researchers need to correctly give statistical results in both the results section and in the figures themselves.
3. Figure 1B. Points in the graph are barely visible.
4. Figure 1C. Add a bar representing the day of the 6hr phase delay.
5. Significance should be highlighted appropriately in the graphs throughout the manuscript and supplemental material.
6. Panel D in Figure 3 should be a separate figure or the graphs should be increased in size. At the moment it is difficult to observe and interpret what is going happening in the individual box and whisker plots. What is the y axis on each of these graphs?
7. The statistical details of the linear regressions for Figure 5A linear regression should be added both in the figures and in the text for these figures.
8. For Figure 5B what were the specific statistical tests performed? How did you account for day-to-day differences given the effects observed when adding leaves?
9. Figure 6A. Please represent significant differences in the plots using brackets and asterisks.
10. Figure 6B. The Daily leaf intake (%) in the light phase seems to be increasing in the light phase of the cycle and is unstable throughout stage I. It seems to me that while the phase delay disrupted this process, it is possible that over time the ants would have reached this steady state on their own. If this is true then why after the phase delay the daily leaf intake seems stable? Same for the dark phase.
11. Especially in light of 10 above - to confirm stable entrainment to a new phase of light cycle the authors should present free-running data in dim red light following Stage 2. This would greatly strengthen the manuscript. In the absence of such data the authors should discuss the specific interpretational limitations this imposes.

Minor comments:

1. Line 50: Jabbur et al., 2021 reference missing from bibliography.
2. Line 56: Missing references
3. Line 305: "To further quantify these changes, we analyzed" incomplete sentence.
4. Line 437: remove the word exciting

Reviewer #3

(Remarks to the Author)

Summary

This study describes a chronobiological experiment conducted using leaf-cutter ants (*Atta sexdens*). The authors report that *A. sexdens* exhibits nocturnal leaf foraging activity and that the colony can re-entrain to a phase shift in the laboratory light cycle after a transient period of around 5 days. I share the authors' opinion that chronobiological work with ants has great potential due to the ability to study biological rhythms at both the individual and collective level. I think that the present manuscript is an excellent contribution to the field of social chronobiology. I do feel that several details of the methods could be more clearly presented through a revision. I also think that some of the paper's results could be better supported with a clearer presentation of the statistical tests used by the authors. That being said, I do think the findings of the paper are interesting and that the authors should be able to address my concerns relatively easily in a revision.

Main comments:

Lines 274-281: I am confused about why the authors are measuring the areas under the curve (AUC). The authors indicate that they want to “quantify the response effect to each leaf replenishment event.”, but I do not see any quantitative analysis of this presented in the results. The authors state that “We visually inspected the AUCs calculated for both leaf-cutting rates and the foraging activity, and we found distinct response effects alongside daily hours for each stage”. Please explain what these distinct responses were and how you identified them. I also do not understand why the authors conducted Shapiro-tests for the AUC measures; I could not find an explanation of this in the methods.

I believe that the authors should be more explicit about the statistical support behind their reported findings. For example, on lines 315-316 of the results they state: “We observed that in Stage I, a higher number of ants were measured crossing the trail in the dark phase.”. However, no statistical information is presented alongside this claim. This claim is essentially just saying that the ants are active nocturnally. I think it is quite clear from the time-series in figure 3 that this is true, but this should definitely be formally tested by the authors.

Similarly, the authors report that “After the 6h phase shift of the LD cycle on day 11, we observe an increase in the proportional daily leaf during the dark phase on the first days, followed by a reversal of this pattern after day 5.”. This is depicted in figure 6B. I may have missed it, but I do not see any explanation of how the authors formally tested if there was a statistically significant shift in leaf intake after the LD phase shift. There are many techniques and R packages for testing for a hypothesized dynamical shift in an empirical time series (for example, the R packages changepoint and mcp are just two possible packages out of several that can be used for this type of changepoint analysis). I think that this type of analysis might be worthwhile for the authors to consider looking into. Although this particular analysis is not essential, I think at least some sort of statistical justification for effect of the LD phase shift is warranted. I also think that there might be a couple of typos here. My suggested correction is as follows: “After the 6h phase shift of the LD cycle on day 11, we observe an increase in the proportional daily leaf intake during the dark phase starting on the first day after the shift, followed by a reversal of this pattern five days later.”.

Lastly, the authors state in the results section on lines 317-319 that: “higher ant frequencies were not related to higher leaf-cutting rates. On the other hand, in the light phase, an increase in the ant flow appears to be proportionally related to an increase in leaf-cutting rates.”. I think the authors should mention that these statements are based on tests of linear correlation. The times series of leaf-cutting rates and number of foraging ants might still exhibit a significant non-linear relationship. Even if the amplitudes of the time series are un-correlated, the timing of the various peaks in the two time-series still might have a non-random phase relationship. This could be directly assessed using various methods, such as mutual information (Quiroz et al. 2002) or by analysing the relative timing of local maxima in the time-series pair (see Cazelles and Stone, 2003 for an example). I do not think it is strictly necessary to conduct such a test, but the authors should at least mention that a non-linear association between the colony's two time-series might be present if they don't explicitly rule one out.

Minor comments:

Line 80: the word “displays” that follows *Atta colombica* should not be italicized.

Line 123: So, the colony's fungal garden was inside the central box in the setup depicted in figure 1A?

Lines 126-128: The feeding protocol is described in the methods as semi-random, but it is not clear to me what this means. Do the authors mean that they used a pseudorandom number generator for each of the two phases of feeding? I think it would be helpful if the authors clarified this. Also, was it not possible to have a large supply of food that would have lasted for the entire experiment? That would have eliminated any concerns about masking effects due to food replenishment.

Line 159: consider replacing the term “food source” with “food box” to standardize the terminology throughout the manuscript.

Line 165: “we measured the leaf area”. Was leaf area computed automatically? Please briefly explain how this was done in FIJI. Based on Figure 2A, it looks like a binary image was created using a thresholding algorithm.

Lines 261-262: “Our video-tracking system allowed us to count a total of 87,671 ants crossing the trail”. Presumably this means 87,671 ant crossing events since the individual identity of ants was not tracked.

Lines 265-266: The authors could consider adding a plot of the periodogram as a supplementary figure. The two time-series that are presented appear to be highly rhythmic, so a visualization of this strong periodicity might be nice, but it is not essential.

Figure 3D: Panel D in Figure 3 does not seem to be referenced anywhere in the main text of the manuscript.

Lines 305-306: This sentence seems to be cut-off. Currently this sentence says: "To further quantify these changes, we analyzed". It seems to end abruptly. The next paragraph starts with the sentence "We analyzed the relationship between leaf-cutting rates and the number of ants foraging, considering both light and dark phases (Figure 5A)." Maybe the authors wanted to just use this sentence instead and forgot to delete the incomplete sentence on lines 305-306?

Figure 4: Please briefly clarify in the caption of figure 4 that the shaded portions of this figure represent times when the lights were off. I noticed that the authors already explain this in caption of figure 3, but I think it would also be useful to include that in the caption for figure 4 as well.

Lines 340-314: The authors state that "Stage II showed a higher daily intake when compared to Stage I ($F=6.3251$, $p=0.01$).". Please specify if this statistical comparison uses the combined data from both the light and dark phases.

References:

Cazelles B, Stone L (2003) Detection of imperfect population synchrony in an uncertain world. *Journal of Animal Ecology* 72:953–968. <https://doi.org/10.1046/j.1365-2656.2003.00763.x>

Quiñan Quiroga R, Kraskov A, Kreuz T, Grassberger P (2002) Performance of different synchronization measures in real data: A case study on electroencephalographic signals. *Phys Rev E* 65:041903. <https://doi.org/10.1103/PhysRevE.65.041903>

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I appreciate the authors' response to my comments. The revised version addresses my previous concerns. However, I found some minor points which need the authors' consideration.

Line 115: 10.000-15.000 individuals should read 10,000-15,000 individuals.

Figure 1B: Please explain what the numbers (0, 05, 10, 15, 20, 25) on each circle mean. Do they indicate time?

Line 243: Please explain what GLMM stands for.

Line 299: 3.23?

Line 309: "with 3 h intervals of day or night" Is this correct?

Figure 4: I think the authors have misused the term Zeitgeber time. Zeitgeber time is not the time of day. It corresponds to the light-dark schedule and Zeitgeber time 0 corresponds to light-on and Zeitger time 12 corresponds to light-off.

Line 379: Stage I

Reviewer #2

(Remarks to the Author)

Revised manuscript COMMSBIO-23-3902A

The revised manuscript titled "The Cost of Re-synchronization in a Leaf-Cutter Ant Colony" addresses all of the reviewers' comments. The figures in the manuscript are now readable and contain accurate statistical information. The authors have added several panels to clarify their analysis methods, included a new table (TABLE I) to address the masking response, and clarified terms where needed. Furthermore, they have discussed the limitations of their experimental setup and data collection pipeline in the Discussion section. Overall, these changes have greatly enhanced the quality and readability of the manuscript and supplemental material. I recommend it for acceptance for publication.

Minor comments:

1. (Optional) Regarding Reviewer 2's comment 11, while the change in language from "entrainment" to "synchronization" addresses the comment, the authors and readers may benefit from adding a sentence or two explaining that this experiment could be a next step and what insights would be gained from it.

2. , lines 368 and 765. Change "Anova" to "ANOVA" in the main text and supplement,

Reviewer #3

(Remarks to the Author)

I think that the authors have done a good job of revising their manuscript; the information in the Results and Methods sections is now presented more clearly. I only have two minor comments on the revised paper, which the authors should be able to very easily address. Apart from the two minor comments below, I have no objections to publishing the revised version of the manuscript.

Lines 343-351 and Figure 5: The authors write that “We analyzed the relationship between leaf-cutting rates and the number of ants foraging, considering both light and dark phases (Figure 5A).”. In my previous review, I pointed out that the authors should present the statistical results of their linear regression analyses (e.g., coefficients of determination and p-values). The authors added the fitted model equations to figure 5A, but the R² and p-values are still missing. This is critical information because the text that references this figure is currently hard to interpret. The authors state that “higher ant frequencies were not related to higher leaf-cutting rates (Figure 5A and 3S).”, but they also claim that “On the other hand, in the light phase, an increase in the ant flow appears to be proportionally related to an increase in leaf-cutting rates.”. These statements appear to be contradictory. Is the flow rate of worker ants correlated with leaf-cutting rates or is it not correlated? Do the authors mean that some of the linear regressions were statistically significant but others were not significant? The new supplementary figure (3S) does not clear up this ambiguity in my opinion. The correlation between ant flow and leaf-cutting rates is a minor part of the overall study, but I still think it would be good for the authors to more clearly present the results of their models, which can be easily done by listing the corresponding R² and p-values.

Figure 4S: Please add units to the x-axis label. I think this should be the period measured in hours?

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REVIEWER 1:

Comments	Author's response
1. The recording for daily activity rhythms was intermittent, taking place every 20 min for 2 min. So data are not available most of time. Therefore, the authors should discuss the validity of their recording and analysis methods.	We chose to do so because video analysis of continuous foraging recording would offer a big computational challenge for any object-detecting machine learning model. However, our recording conditions offer 72 time points for each rhythmic variable every 24h (higher than several sample collections common in rhythmic analysis of behavioral or physiological variables found in literature). Included in line 179-180: "Even though we used intermittent data collection, its sampling frequency was sufficient for rhythmic data analyses of the colony."
2. Regarding the recordings, they state that the record was performed for 120 min before and after leaf replenishments when analyze the masking effects of leaf replenishments. However, they do not state how they analyzed the data. They should explain the method appropriately. For example, what was the time span unit for the analysis?	We appreciate these comments regarding our AUC analysis, as they showed us that our explanations were not clear. To calculate the short-term masking responses, we measured the area under the curve (AUC) of each variable 120 min before and after leaf replenishments. These 120 min intervals correspond to 6 intervals with 20min each. The AUC was thus calculated by dividing the total area into 6 smaller trapezoids and applying the "trapezoidal rule of integration" which sums all trapezoid sub-areas. Finally, to evaluate the masking effects, we calculated the ratio (Short Term Masking Response) between the AUC after and AUC before leaf replenishment. We added a table (Table I) with all the ratios for Stage I, a new figure (2D), changed the way we presented them in Figure 3BC, and added explanations of the methods, the analysis, and our interpretations. Figure 2 legend: L204-208. Methods: L220-225; L241-244; Results: L281-283; L285-292; L295-307. Discussion: L409-415

3. L118: The materials of the colony maze should be mentioned. Is this made of transparent plastic? Can light penetrate into the maze? Are all ants exposed to light during the light phase? These are very important information for the entrainment experiment.	The maze was made of translucent plastic. Line 119-120: "plastic maze (translucent)." Line 124-125: "All ants were exposed to these light conditions".
4. L233: Please explain what is AUC? How did you calculate AUC?	Answered in point 2.
5. L270: influenced ant rhythms	Sentence removed.
6. L280: Here you should show actual value of AUCs then you should show the result of statistical tests. You should clearly describe which values were statistical compared.	We included the actual values of the Short term masking responses (AUC after/AUC before) in Table I and further explanation about this analysis and results in several parts of the paper, as listed in 2. Changes made on lines: L247-251 and L285-292, "Additionally, we performed a GLMM analysis for the variables foraging and leaf-cutting ratios, comparing the actual data points from before and after leaf replenishments (before-after factor) and days when feeding occurred in the light (days 5, 7, 8, and 10) or dark (days 3, 4, 6, and 9) phases (light-dark factor), as well as the interaction between these two." "Our analysis shows that foraging is lower in the light phase after leaf replenishments when compared to the dark phase (GLMM, $z = -3.553$, $p < 0.001$) and lower before feeding events (GLMM, $z = -26.661$, $p < 0.001$). Regarding the leaf-cutting rates, values are lower in the light phase (GLMM, $z = -3.299$, $p < 0.001$) and lower before feeding events (GLMM, $z = -7.608$, $p < 0.001$). The interaction of these factors shows a marginally significant increase in leaf-cutting (GLMM, $z = 1.712$, $p = 0.08$). We calculated the AUC ratio for each day after the leaf offering and used this to quantify the short-term masking response (STR) effect of each leaf replenishment event (Table I)."
7. L306: This sentence is uncompleted. There is no description on data shown in Figure 4.	Thank you for pointing that out. Over the corrections, we accidentally deleted part of

	this sentence. Correction made between L335-L339.
8. Figure 3A: Please add label of abscissa. Ordinate labels should be Foraging and Leaf cutting rate.	Figure 3A was edited accordingly.
9. Figure 3BC: Time of day on the top of each panel should be deleted. Instead the time of leaf replenishment is better. Please move label of abscissa to an appropriate place.	Figure 3BC was edited accordingly.
10. Figure 3D: average daily rhythms: How many days did you use for the average? Especially for Stage II, what are the days? Why do you include Stage II data here? Ordinate labels should be provided.	We changed this figure and it currently shows average estimated values for foraging and leaf-cutting rhythms obtained by the GLMM analysis mentioned in 2.5.1 General rhythmicity and masking effects. For this analysis, we used only <i>Zeitgeber</i> time and included all days of Stage I and only synchronized days of Stage II (days 16-20). Changes made in: L220-226. “We also performed generalized linear mixed models to observe if our variables (number of ants and leaf-cutting rates) showed a rhythmic pattern according to the <i>zeitgeber</i> time before and after the phase shift (categorical predictors were <i>zeitgeber</i> time and stages, the interaction between these two; days of the experiment were included in the model as random effects). We only included synchronized days for the Stage II (days 16-20). For both variables, our minimum adequate model included all the predictors mentioned above (Figure 4B).”
11. Line 324: “As of the 6th day of stage II (days 16 to 20)”: This is very confusing.	Change made on line L364: “As of day 16 (6th day of Stage II)”
12. Line 324: “we recovered the pattern described for stage I” Please consider to say the pattern observed in Stage I was restored.	Change made on line L364: “restored”

13. Line 326: This sentence is incomplete.	Change made on lines L365-366. "We considered therefore that the colony resynchronized after 5 days of transients after the phase shift of the LD cycle (Figure 5A)."
14. L314:- The authors examined the correlations between leaf cutting rates and ant flow and compared them between stages. However, no actual values and significance were stated. These should be clearly mentioned.	We added the equations for each regression model in figure 5A and a supplementar figure showing the comparisons between each linear models for the light and dark phase for each plot in figure 5A.
15. L328: Please consider to say "Daily changes in the ratio between...."	Change made on L367.
16. L342: P=0.07 shows not significant.	Change made on L383-384: "Leaf intake seemed higher during the dark phase during Stage I, as we found a marginally significant difference (F=3.482, p=0.07)."
17. L348: daily leaf intake in the dark phase during the first 5 days	Change made after combining all reviewers suggestions. L390-392: "After the 6h phase shift of the LD cycle on day 11, we observe an increase in the proportional daily leaf intake during the dark phase starting on the first day after the shift, followed by a reversal of this pattern five days later (after day 15)."
18. L349: after day 15.	Change made. L392.
19. L354: insert period after II.	Change made. L397.
20. L368: Why could you say the colony is particularly sensitive near 0h? In Fig. 3B, there is no strong evidence for this statement. There is no statement in the Results section regarding this point. The authors should describe important points of the effects of leaf replenishment in the results section.	In addition to what was discussed in point 2, we used the values of STR (Short-term masking responses) to interpret colony sensitivity. We combined these values with aspects of the colony's behavior to discuss that the colony was particularly sensitive to changes near 18h (higher STRs for both foraging and leaf-cutting activity) and also near 0h, as this is the peak of colony activity and still has high proportional increases in both variables, which correspond to the highest absolute values of foraging and leaf-cutting rates by the colony. Change made in L409-415.

21. L327: "Our results indicated..." I don't understand the meaning of this sentence.	This sentence was removed.
22. L372-282: In this paragraph, the authors discussed the re-entrainment to the delayed LD and concluded the re-entrainment does not complete within 10 days after the shift. However, in other places, they discussed their results assuming that the re-entrainment is accomplished during the first 5 days of the shift. When look at Figure 3D, the daily distribution of activity is quite similar between Stage I and II, suggesting complete re-entrainment of the rhythms. So the discussion is quite confusing to this reviewer. Resynchronization and criteria for determining re-entrainment needs to be properly defined.	We agree that these results can be confusing. Our interpretations of re-synchronization and re-entrainment are adapted to the colony context and the different levels where synchronization could take place. Regarding collective behavior aspects (patterns of foraging and leaf-cutting), we recover the rhythms observed in Stage I after 5 days in Stage II (GLMM analysis in Figure 4B), including the patterns of Short-Term Masking Response (See supplementary material). In that sense, as the rhythms of foraging and leaf-cutting at the colony level were recovered, we were surprised to find that the pattern of % Daily leaf intake (Figure 6B) was completely different from what we observed in Stage I. Since re-entrainment would assume that all the relationships at the inner level of the organization were also recovered, we raised the possibility that the colony, even though showing re-entrained behaviors, was not completely re-synchronized. The ant colony is a complex system and foraging and leaf-cutting activity can be extremely dynamic, so it is also possible that re-synchronization could be possible with different patterns of organization within the colony, however, our experiments are not able to distinguish between both explanations. Our data and results show re-entrainment at a colony level, but it seems that this happens without recovering the exact same organization dynamics within the colony, thus we try to discuss both possibilities. We made a few changes in the discussion to clarify these points. L420-426: "Although we observed colony-level re-synchronization after 5 days (Figures 4, 5A, and 1S, 2S in the supplementary materials) however, deeper analysis of the internal dynamics of the organization revealed that we never recovered exactly the same patterns of daily proportional leaf intake (Figure 6B). We applied a 6h phase delay of

	the LD cycle, assuming that a 10-day interval between these perturbations was sufficient for full resynchronization (complete recovery of patterns of foraging and leaf-cutting rhythms and recovery of the organization patterns within colony).”
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REVIEWER 2:

Comments	Author's response
1. The Introduction needs a section speaking about the basic biology of <i>Atta sexdens</i> . This would help readers who are not familiar with <i>Atta sexdens</i> and their life history. For example, the importance of fungi for these ants was first mentioned in the discussion section.	Changes made between L93-100. “We chose to perform this study using <i>Atta sexdens</i> colonies, as leaf-cutter ants are vastly distributed over South and Central America and Mexico, show economic importance related to agricultural activities, and are characterized by a gradual polymorphism, allowing easier identification of ant castes. They cultivate a garden of the fungus <i>Leucoagaricus gongylophorus</i> with which they formed a symbiotic relationship (Silva et al., 2003). <i>Atta sexdens</i> colonies reach 8 million individuals in nature and hundreds of thousands of individuals under laboratory conditions.”
2. While the statistical methods mentioned in the methods section are appropriate, the researchers need to correctly give statistical results in both the results section and in the figures themselves.	Edits made in figures 5A and 6A. Regarding AUCs analysis, we included a table showing the actual values of Short-term masking responses (AUC after feeding/ AUC before feeding). We also included a GLMM analysis for the variables foraging and leaf-cutting ratios, comparing the actual data points shown in Figure 3BC from before and after leaf replenishments (before-after factor) and days when feeding occurred in the light (days 5, 7, 8, and 10) or dark (days 3, 4, 6, and 9) phases (light-dark factor), as well as the

	interaction between those two factors. For the foraging variable, our minimum adequate model contained both before-after and light-dark factors, but not the interaction between both. For the leaf-cutting rates variable, our minimum adequate model contained these two factors and also the interaction between them. Several different edits were made: Figure 2 legend: L204-208. Methods: L220-225; L241-244; Results: L281-283; L285-292; L295-307. Discussion: L409-415
3. Figure 1B. Points in the graph are barely visible.	Edit made.
4. Figure 1C. Add a bar representing the day of the 6hr phase delay.	Edit made.
5. Significance should be highlighted appropriately in the graphs throughout the manuscript and supplemental material.	Edit made where we found appropriate.
6. Panel D in Figure 3 should be a separate figure or the graphs should be increased in size. At the moment it is difficult to observe and interpret what is going happening in the individual box and whisker plots. What is the y axis on each of these graphs?	We appreciate the comments. Edits were made to make the figures bigger and we labeled all axes. We also transformed it in Figure 4B, since it seemed more appropriate. This figure currently shows average estimated values for foraging and leaf-cutting rhythms obtained by the GLMM analysis mentioned in 2.5.1 General rhythmicity and masking effects. For this analysis, we used only Zeitgeber time and included all days of Stage I and only synchronized days of Stage II (days 16-20).
7. The statistical details of the linear regressions for Figure 5A linear regression should be added both in the figures and in the text for these figures.	We added the equations for each regression model in Figure 5A and a supplementary figure showing the comparisons between each linear model for the light and dark phase for each plot in Figure 5A.
8. For Figure 5B what were the specific statistical tests performed? How did you account for day-to-day differences given the effects observed when adding leaves?	We performed one-way ANOVAs. First, we compared the whole experiment how efficiency changed between light and dark phases. Then we compared the differences between Stage I and II. The novelty effect (when we introduce new leaves - described by Roces and Holldobler, 1996) has a Short

	term masking response (a couple of hours) and the efficiency is analyzed considering the daily time scale. L351-357 and L365-366.
9. Figure 6A. Please represent significant differences in the plots using brackets and asterisks.	Edit made on Figure 6A.
10. Figure 6B. The Daily leaf intake (%) in the light phase seems to be increasing in the light phase of the cycle and is unstable throughout stage I. It seems to me that while the phase delay disrupted this process, it is possible that over time the ants would have reached this steady state on their own. If this is true then why after the phase delay the daily leaf intake seems stable? Same for the dark phase.	We believe that happens due to the amount of days included in the analysis. Overall we found significant differences in leaf intake comparing Stage I and II, and the light and dark phases of Stage I (Figure 6A). We think that the reason we could not find significant differences in daily leaf intake between light and dark phases of Stage II is due to the shift of the pattern described in Figure 6B.
11. Especially in light of 10 above - to confirm stable entrainment to a new phase of light cycle the authors should present free-running data in dim red light following Stage 2. This would greatly strengthen the manuscript. In the absence of such data the authors should discuss the specific interpretational limitations this imposes.	We agree and appreciate the suggestion. Unfortunately, we are not capable of performing further experiments in the short term. We acknowledge that this experimental step (release into constant conditions) that enables discrimination of synchronization by entrainment from synchronization by masking (Moore-Ede et al., 1982) was not made. Therefore we substituted “entrainment” and “reentrainment” by the more general term, “synchronization” and “resynchronization” which refers broadly to the achievement of a fixed phase relationship between the LD cycle and the rhythm.
MINOR COMMENTS	
1. Line 50: Jabbur et al., 2021 reference missing from bibliography.	Correction made on line 48 and 629-630. “Jabbur and Johnson, 2022: Spectres of Clock Evolution: Past, Present, and Yet to Come. Doi: 10.3389/fphys.2021.815847. “
2. Line 56: Missing references	Correction made on L54: (Fuchikawa, 2023).

	We also added Dolai et al., 2024 on L78-79: "Rain and predation cause a diminution of circadian foraging activity in Asian weaver ant <i>Oecophylla smaragdina</i> (Dolai et al., 2024)."
3. Line 305: "To further quantify these changes, we analyzed" incomplete sentence.	Corrected between lines 336-340.
4. Line 437: remove the word exciting	Change made on L485: "rates may be a compelling measure of system"

REVIEWER 3:

Comments	Author's response
Lines 274-281: I am confused about why the authors are measuring the areas under the curve (AUC). The authors indicate that they want to "quantify the response effect to each leaf replenishment event.", but I do not see any quantitative analysis of this presented in the results. The authors state that "We visually inspected the AUCs calculated for both leaf-cutting rates and the foraging activity, and we found distinct response effects alongside daily hours for each stage". Please explain what these distinct responses were and how you identified them. I also do not understand why the authors conducted Shapiro-tests for the AUC measures; I could not find an explanation of this in the methods.	We appreciate these comments regarding our AUC analysis, as they showed us that our explanations were not clear. To calculate the short-term masking responses, we measured the area under the curve (AUC) of each variable 120 min before and after leaf replenishments. These 120 min intervals correspond to 6 intervals with 20min each. The AUC was thus calculated by dividing the total area into 6 smaller trapezoids and applying the "trapezoidal rule of integration" which sums all trapezoid sub-areas. Finally, to evaluate the masking effects, we calculated the ratio STR between the AUC after and AUC before leaf replenishment. We added a table (Table I) with all the ratios for Stage I, a new figure (2D), changed the way we presented them in figure 3BC, and added explanations of the methods, the analysis, and our interpretations. We additionally performed a GLMM analysis for the variables foraging and leaf-cutting ratios, comparing the actual data points shown in Figure 3BC from before and after leaf replenishments (before-after factor) and days when feeding occurred in the light (days

	5, 7, 8, and 10) or dark (days 3, 4, 6, and 9) phases (light-dark factor), as well as the interaction between those two factors. For the foraging variable, our minimum adequate model contained both before-after and light-dark factors, but not the interaction between both. For the leaf-cutting rates variable, our minimum adequate model contained these two factors and also the interaction between them Figure 2 legend: L204-208. Methods: L220-225; L241-244; Results: L281-283; L285-292; L295-307. Discussion: L409-415
I believe that the authors should be more explicit about the statistical support behind their reported findings. For example, on lines 315-316 of the results they state: “We observed that in Stage I, a higher number of ants were measured crossing the trail in the dark phase.”. However, no statistical information is presented alongside this claim. This claim is essentially just saying that the ants are active nocturnally. I think it is quite clear from the time-series in figure 3 that this is true, but this should definitely be formally tested by the authors.	Change made on L354-357: “We observed that in Stage I, a higher number of ants were measured crossing the trail in the dark phase (ANOVA, $F=299.69$, $p<0.0001$).”
Similarly, the authors report that “After the 6h phase shift of the LD cycle on day 11, we observe an increase in the proportional daily leaf during the dark phase on the first days, followed by a reversal of this pattern after day 5.”. This is depicted in figure 6B. I may have missed it, but I do not see any explanation of how the authors formally tested if there was a statistically significant shift in leaf intake after the LD phase shift. There are many techniques and R packages for testing for a hypothesized dynamical shift in an empirical time series (for example, the R packages changepoint and mcp are just two possible packages out of several that can be used for this type of changepoint analysis). I think that	We clarified our results using a GLMM test (Figure 4B). Change made combining all reviewers' suggestions: L390-392: “After the 6h phase shift of the LD cycle on day 11, we observe an increase in the proportional daily leaf intake during the dark phase starting on the first day after the shift, followed by a reversal of this pattern five days later (after day 15).”

this type of analysis might be worthwhile for the authors to consider looking into. Although this particular analysis is not essential, I think at least some sort of statistical justification for effect of the LD phase shift is warranted. I also think that there might be a couple of typos here. My suggested correction is as follows: “After the 6h phase shift of the LD cycle on day 11, we observe an increase in the proportional daily leaf intake during the dark phase starting on the first day after the shift, followed by a reversal of this pattern five days later.”.	
Lastly, the authors state in the results section on lines 317-319 that: “higher ant frequencies were not related to higher leaf-cutting rates. On the other hand, in the light phase, an increase in the ant flow appears to be proportionally related to an increase in leaf-cutting rates.”. I think the authors should mention that these statements are based on tests of linear correlation. The times series of leaf-cutting rates and number of foraging ants might still exhibit a significant non-linear relationship. Even if the amplitudes of the time series are un-correlated, the timing of the various peaks in the two time-series still might have a non-random phase relationship. This could be directly assessed using various methods, such as mutual information (Quián Quiroga et al. 2002) or by analysing the relative timing of local maxima in the time-series pair (see Cazelles and Stone, 2003 for an example). I do not think it is strictly necessary to conduct such a test, but the authors should at least mention that a non-linear association between the colony’s two time-series might be present if they don’t explicitly rule one out.	We thank the reviewer for pointing this out and agree. Clarification made between L355-357. “Although we cannot rule out non-linear relationships, our linear correlation analyses indicate that these higher ant frequencies were not related to higher leaf-cutting rates (Figure 5A and 3S-Supplementary Material).”
MINOR COMENTS	

Line 80: the word "displays" that follows <i>Atta colombica</i> should not be italicized.	Correction made on Line 80.
Line 123: So, the colony's fungal garden was inside the central box in the setup depicted in figure 1A?	Yes. We made some changes on lines 97-98 about the biology of <i>Atta sexdens</i> that might help make that more clear. When we mention the colony, that includes the fungus garden and the ants. Change made on L119. "we housed the colony (ants and fungus garden)"
Lines 126-128: The feeding protocol is described in the methods as semi-random, but it is not clear to me what this means. Do the authors mean that they used a pseudorandom number generator for each of the two phases of feeding? I think it would be helpful if the authors clarified this. Also, was it not possible to have a large supply of food that would have lasted for the entire experiment? That would have eliminated any concerns about masking effects due to food replenishment.	A large food supply would indeed be ideal. Unfortunately, leaf-cutter ants show a strong preference for fresh leaves, called "novelty effect" (Roces and Holldobler, 1996). If the leaves are not fresh enough (more than a day), they stop cutting it. To ensure they were not food deprived or stressed, and also to have the possibility of easily recording the leaf-cutting activity, we randomized the timing of feeding protocol . We had described the feeding protocol as "semi-random" due to the two stages described in item 2.2 Feeding protocol but we think now that we can refer to it as random. Correction made on the term.
Line 159: consider replacing the term "food source" with "food box" to standardize the terminology throughout the manuscript.	Change made. Line 160.
Line 165: "we measured the leaf area". Was leaf area computed automatically? Please briefly explain how this was done in FIJI. Based on Figure 2A, it looks like a binary image was created using a thresholding algorithm.	Change made on L167-169: "We created a binary image file using the default thresholding algorithm in FIJI. After that, we selected the corresponding area that contained the leaves and standardized the measure to centimeters."
Lines 261-262: "Our video-tracking system allowed us to count a total of 87,671 ants crossing the trail". Presumably this means 87,671 ant crossing events since the individual identity of ants was not tracked.	We thank the reviewer for pointing this out. In fact, the colony had a total of 10.000-15.000 thousand individuals. Corrected on L274-276 . "Our video-tracking system allowed us to count a total of 87,671 ant crossing events in

	the trail, even though our software validation process shows that our software tends to underestimate the total number of ants in each video (Figure 2C)."
Lines 265-266: The authors could consider adding a plot of the periodogram as a supplementary figure. The two time-series that are presented appear to be highly rhythmic, so a visualization of this strong periodicity might be nice, but it is not essential.	We appreciate the reviewer's suggestion. We added the periodograms in our supplementary materials.
Figure 3D: Panel D in Figure 3 does not seem to be referenced anywhere in the main text of the manuscript.	Change made: L403-405: "Our experiment showed clearly that daily and synchronized activity rhythms at the colony level could be reproduced in a colony maintained under artificial LD cycles and constant temperatures under laboratory conditions (Figure 4B)."
Lines 305-306: This sentence seems to be cut-off. Currently this sentence says: "To further quantify these changes, we analyzed". It seems to end abruptly. The next paragraph starts with the sentence "We analyzed the relationship between leaf-cutting rates and the number of ants foraging, considering both light and dark phases (Figure 5A)." Maybe the authors wanted to just use this sentence instead and forgot to delete the incomplete sentence on lines 305-306?	We appreciate the correction. Change made between lines 336-340.
Figure 4: Please briefly clarify in the caption of figure 4 that the shaded portions of this figure represent times when the lights were off. I noticed that the authors already explain this in caption of figure 3, but I think it would also be useful to include that in the caption for figure 4 as well.	Correction made in the caption. L345-346: "Grey areas represent the dark phase, while white areas correspond to the light phase of the LD cycle".
Lines 340-314: The authors state that "Stage II showed a higher daily intake when compared to Stage I ($F=6.3251$, $p=0.01$).". Please specify if this statistical comparison	Brackets and asterisks included in figure 6A. Correction made on L381-383: "Stage II showed a higher daily intake when compared to Stage I (light and dark phases included,

uses the combined data from both the light and dark phases.

$F=6.3251, p=0.01).$ ”

We thank the reviewers for their comments, constructive criticisms and suggestions. We hope we have addressed all their concerns.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

I appreciate the authors' response to my comments. The revised version addresses my previous concerns. However, I found some minor points which need the authors' consideration.

Line 115: 10.000-15.000 individuals should read 10,000-15,000 individuals.

We appreciate for pointing this typing error. Correction made.

Figure 1B: Please explain what the numbers (0, 05, 10, 15, 20, 25) on each circle mean. Do they indicate time?

They correspond to the default grid labels from the clock24.plot function. We removed them from the last version to avoid confusion by the reader.

Line 243: Please explain what GLMM stands for.

Correction made.

Line 299: 3.23?

Correction made.

Line 309: "with 3 h intervals of day or night" Is this correct?

*We clarified this part as the following: "**B-C**) Dependence of short-term masking responses to feeding events at that time of the day. Each graph row is composed of eight graphs showing 120 minutes before and after leaf replenishment (point 0 on the x-axis). The background color (light or dark) and position of the graph indicate the temporal order that leaf replenishment took place. Days and local time of leaf replenishments are shown on the top of each graph."*

Figure 4: I think the authors have misused the term Zeitgeber time. Zeitgeber time is not the time of day. It corresponds to the light-dark schedule and Zeitgeber time 0 corresponds to light-on and Zeitger time 12 corresponds to light-off.

We believe the use of Zeitgeber time was accurate because we compared the rhythmicity between Stages I and II which are 6h apart on a common x-axis. To do so, we used zeitgeber time (light from 06:00-18:00 local time during Stage I and from 12:00 to 00:00 local time during Stage II).

Line 379: Stage I

Correction made.

Reviewer #2 (Remarks to the Author):

Revised manuscript COMMSBIO-23-3902A

The revised manuscript titled "The Cost of Re-synchronization in a Leaf-Cutter Ant Colony" addresses all of the reviewers' comments. The figures in the manuscript are now readable and contain accurate statistical information. The authors have added several panels to clarify their analysis methods, included a new table (TABLE I) to address the masking response, and clarified terms where needed. Furthermore, they have discussed the limitations of their experimental

setup and data collection pipeline in the Discussion section. Overall, these changes have greatly enhanced the quality and readability of the manuscript and supplemental material. I recommend it for acceptance for publication.

Minor comments:

1.(Optional) Regarding Reviewer 2's comment 11, while the change in language from "entrainment" to "synchronization" addresses the comment, the authors and readers may benefit from adding a sentence or two explaining that this experiment could be a next step and what insights would be gained from it.

We thank the reviewer for the suggestion and added the following in the Discussion:

"In a future study, it would be important to release the colony into constant darkness following maintenance in an LD cycle. This would further prove that the observed rhythm is endogenous and not a reaction to the presence or absence of light. Furthermore, release into constant darkness after the phase shift of the LD cycle will discriminate between synchronization due to entrainment or to masking (Moore-Ede et al, 1982)."

2.lines 368 and 765. Change "Anova" to "ANOVA" in the main text and supplement.

Changes made.

Reviewer #3 (Remarks to the Author):

I think that the authors have done a good job of revising their manuscript; the information in the Results and Methods sections is now presented more clearly. I only have two minor comments on the revised paper, which the authors should be able to very easily address. Apart from the two minor comments below, I have no objections to publishing the revised version of the manuscript.

Lines 343-351 and Figure 5: The authors write that "We analyzed the relationship between leaf-cutting rates and the number of ants foraging, considering both light and dark phases (Figure 5A)". In my previous review, I pointed out that the authors should present the statistical results of their linear regression analyses (e.g., coefficients of determination and p-values). The authors added the fitted model equations to figure 5A, but the R² and p-values are still missing. This is critical information because the text that references this figure is currently hard to interpret. The authors state that "higher ant frequencies were not related to higher leaf-cutting rates (Figure 5A and 3S).", but they also claim that "On the other hand, in the light phase, an increase in the ant flow appears to be proportionally related to an increase in leaf-cutting rates.". These statements appear to be contradictory. Is the flow rate of worker ants correlated with leaf-cutting rates or is it not correlated? Do the authors mean that some of the linear regressions were statistically significant but others were not significant? The new supplementary figure (3S) does not clear up this ambiguity in my opinion. The correlation between ant flow and leaf-cutting rates is a minor part of the overall study, but I still think it would be good for the authors to more clearly present the results of their models, which can be easily done by listing the corresponding R² and p-values.

We appreciate the reviewer comment and added R² and p -values to figure 5A. We also changed the text on lines 342-349 to: “ We analyzed the relationship between leaf-cutting rates and the number of ants foraging, considering both light and dark phases (Figure 5A). Comparisons between linear models between light and dark phases for each plot are shown in supplementary material (Figure 3S). We observed that in Stage I, a higher number of ants were measured crossing the trail in the dark phase (ANOVA, F=299.69, p<0.0001). Although we cannot rule out non-linear relationships, our linear correlation analysis indicate that these higher ant frequencies were not related to higher leaf-cutting rates in the same relationship observed during the light phase (Figure 5A and 3S). In the light phase, an increase in the ant flow appears to be proportionally related to an stronger increase in leaf-cutting rates.”

Figure 4S: Please add units to the x-axis label. I think this should be the period measured in hours?

Change made.

We thank all the reviewers for the suggestions and we hope the manuscript is suited for publication in Communications Biology.