molecular systems biology

Network balance *via* CRY signalling controls the Arabidopsis circadian clock over ambient temperatures

Peter D. Gould, Nicolas Ugarte, Mirela Domijan, Maria Costa, Julia Foreman, Dana MacGregor, Ken Rose, Jayne Griffiths, Andrew J.Millar, Bärbel Finkenstädt, Steven Penfield, David A. Rand, Karen J. Halliday, Anthony J.W. Hall

Corresponding author: Anthony J.W. Hall, University of Liverpool

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 08 March 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the four referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees raise substantial concerns on your work, which, I am afraid to say, preclude its publication.

The four reviewers, in general, appreciated the goals of this work, but they raised a series of issues that they felt raised important doubts about the conclusiveness of this work, at present. In particular, the reviewers indicated that the temperature compensation model presented here required more thorough and direct validation. They also felt that this model needs to be placed more completely in the context of previous works (Gould et al, 2006; Salome et al, 2010) and apparent discrepancies in the results need to be thoroughly investigated. They also raised a series of specific, but fundamental concerns, such as possible temperature dependent activity of cryptochromes, which they felt could provide alternative explanations for these results.

GIven the extensive nature of these concerns, we feel we have no choice but to return this manuscript with the message that we cannot offer to publish it.

Nevertheless, the reviewers did express interest in the topic of this work and the approach employed. Moreover, they each recommend a series of additional experiments that could potentially help to

address their concerns. As such, I would like to suggest that we may be willing to consider a new, substantially expanded submission based on this work. Any such resubmission would need to include substantial additional experimental work that addresses the concerns raised by these reviewers, and provides further direct support for the temperature compensation model described here. Of note, three reviewers independently indicated that analyses of phytochrome mutants would be essential, and the first reviewer felt that experiments at 22C would be needed to help reconcile these results with previous publications. Given that these additional experiments remain rather substantial, we would understand if you decided instead to submit this work to another journal.

A resubmitted work would have a new number and receipt date. As you probably understand, we can give no guarantee about its eventual acceptability. If you do decide to follow this course then it would be helpful to enclose with your re-submission an account of how the work has been altered in response to the points raised in the present review.

I am sorry that the review of your work did not result in a more favorable outcome on this occasion, but I hope that you will not be discouraged from sending your work to Molecular Systems Biology in the future.

Thank you for the opportunity to examine this work.

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors wished to explore the basis for temperature compensation in Arabidopsis, with the use of luciferase reporters for two output genes, CAB2 and CCR2. They measured free-running period following entrainment by light-dark cycles in a number of temperatures (12, 17 and 27∫C) and genotypes (Col-0, cry1-304, cry2-1, cry1-304 cry2-1). They found that all genotypes displayed overcompensation (period lengthens with increasing temperature) in red light, indicating that crys might play a role in temperature compensation. The authors then attempt to adjust their previous model from 2010 to include this new piece of data. The new model predicts that LHY protein levels should increase with temperature, and they do, demonstrating that their latest model accurately reflects the experimental data.

I have a number of concerns about the data, the way they were analyzed, and some of the conclusions, as listed below. Overall, the manuscript could be made clearer, both in the text and figures.

1. I know it sounds good, but you did not have 90,000 useable data points as mentioned on page 6. You cannot do anything with the single LUC activity data of single time-points, so I would like this statement deleted. Just say how many period values you generated (1900 or so, as mentioned on top of page 7).

2. I imagine only half of the data collected on photoreceptor mutants is being shown here: why not report the data on phy mutants (phyA, phyB and phyA phyB) that the authors must have collected?

3. in the Devlin and Kay article (Plant Cell 2000. 12: 2499) a cry1cry2 double mutant had a long period for fluence rates of red light ~40 µmol/sec/m2 and below. I realize this was at 22∫C, but Figure 1C in this manuscript shows very little effect of red light in the cry1cry2 double mutant. Yet, on page 7, 5 lines before the end of the page, the authors state that their results agree with Devlin and Kay.

4. What is the fluence rate being used here, for red and blue light, alone and combined? This might in part explain the above discrepancy, if red light fluence rates were above 50 µmol/sec/m2 (which would be quite high). This could also explain why cry2-1 has almost no effect on period: fluence is too high.

5. It is a shame not to have included data points at 22∫C. This generates a big gap in data, from 17∫C to 27∫C, and prevents direct comparison with others' results.

6. About the analysis: in my mind, 96 hours is too short. 120 hours would have been preferable, especially because I would start the analysis only on ZT12 on the first day in LL for CAB2, and even later for CCR2. One of the reasons RAEs are so high at 27∫C for CCR2 in blue light is likely to because of the first peak in LL, which has a higher amplitude. RAEs are informative in describing strength of rhythm, but only when amplitude does not vary too much. RAE only measure the fit to the sine wave fitted by FFT-NLLS. I think FFT-NLLS latches on to this first peak and fits the sine wave to it, to the detriment of the remaining cycles.

8. the symbols in Figure 1 are too big and too numerous, so one cannot tell them apart. Since the authors make no comments about the direction of RAEs vs period length, I would redo the graphs as box or dot plots for RAE data only; the period data are already shown in Figure 1C.

9. It would be very useful to see the mean traces before normalization, for a representative transgenic line.

10. what were the traces normalized to? Mean LUC activity over the whole time-course for each seedling?

11. I would also like to see traces for cry1cry2 at 27∫C in blue light, for the arrhythmic set and for the rhythmic set.

12. 38% to 44% of cry1cry2 seedlings are still rhythmic in blue light at 27∫C, and display a normal period length. This does not qualify as strongly arrhythmic, unlike an elf3 mutant for example. Can the model explain the remaining rhythmicity?

13. the last sentence of the first paragraph on page 8 bothers me. For one, it is too early to make this suggestion based on the data thus far. Second, it skirts around an issue that admittedly took me a while to formulate. The Somers and Devlin articles on circadian entrainment by photoreceptors looked at free-running period under a range of fluences at a single temperature. The authors here (and others before) measured free-running period at a single fluence and over a range of temperatures. In both cases, seedlings were first entrained to LD cycles, then released in constant conditions. In both assays, the seedlings are thus grown under the same conditions.
When does one decide that a change in period reflects a problem with entrainment of the clock vs compensation of the clock? This is rather important since the authors claim that compensation goes through photoreceptors, and thus circadian entrainment will be affected.

14.the authors used LUC reporters for output genes, and they might therefore not reflect what the clock is really doing. Another reporter, like CCA1:LUC or TOC1:LUC or PRR9:LUC, all used in the 2010 Pokhilko article, would be a lot better. Data from Figure 2 could be used to infer the behavior of Col-0 at the various temperatures in blue light, but not for cry1cry2.

15. in Figure 2, I would argue that all clock genes are arrhythmic in cry1cry2 at all temperatures in the experimental dataset, but not in the model. In fact, the model appears to perform poorly at all temperatures when predicting clock gene expression in the double mutant.

16. all models to date have had TOC1 as a positive regulator of CCA1/LHY expression. However, the latest data from the Kay lab shows that TOC1 is actually a transcriptional repressor. I know the Kay article was published online on February 6, so about one week after this manuscript was submitted. However, one of the co-authors (Karen Halliday) attended the 2011 Photobiology meeting in China, during which Steve presented these results. Given how badly the model seems to be at predicting clock gene expression in cry1cry2, wouldn't it be better to try and figure out why none of the models anticipated the repressor role of TOC1, even though all models were presumably based on experimental data?

17. the authors state in this manuscript that the parameters in their 2010 model (at 22∫C in combined red and blue light) are similar to the experimental data they collected here in blue light at 27∫C. This likely is due to the absence of data in this manuscript at 22∫C. Again, it would have been very nice to collect data at 22∫C, for no other reason than to extend the 2010 model. I think this contributes to the poor performance of the new model.

18. are LHY protein levels changing in the cry1cry2 double mutant between 17∫C and 27∫C? And what happens at 22∫C in both Col-0 and the double mutant?

19. cryptochromes are kinases, and therefore enzynes. Couldn't their kinase activity be temperature-dependent?

20. Indeed, crys are not known to interact with any of the clock proteins. This only means that they never showed up in a general 2-hybrid screen. But the TOC1/ZTL interaction only came from a directed 2-hybrid, so it would be worth looking into this. cry1 autoactivates, but it can still be used as prey against the other clock proteins.

21. I have never seen this discussed anywhere before, but luciferase is also an enzyme, so its activity will change with temperature. This should be taken into account when comparing amplitudes between different temperature treatments.

22. the fourth sentence of the introduction should say "unusually temperature-independent", not "unusually temperature-dependent".

23. on page 12, first paragraph of discussion: although cry1cry2 mutants remain rhythmic in blue light, this is not the case for the phy quintuple mutant in red light.

24. arrhythmia should be changed to arrhythmicity.

25. all seedlings were first entrained with LD cycles comprising of white light. When relative to ZT0 or ZT12 were the seedlings transferred to blue, or red, or blue + red?

Reviewer #2 (Remarks to the Author):

In this manuscript the authors analyze the effect of light on the temperature compensation process of the Arabidopsis circadian clock. The authors observe a strong period lengthening effect at higher temperatures under red light in wild type seedlings but not under blue light. They also find that cryptochromes play a role in temperature compensation under blue and blue/red light, and a small effect under red light. Using a mathematical model of the plant circadian clock published previously they showed that introducing temperature dependency only in some light regulated processes is sufficient to simulate temperature compensation. One of the predictions of the temperature compensated model was that the LHY protein levels would increase at higher temperatures. The authors then show that in plants LHY protein levels significantly increase at 27C when compared to 12C.

The author's argue that based on their modeling and experimental results
" light-responsive processes were the most important temperature inputs". However, several other clock mutants display similar or stronger effects on temperature compensation as described by the authors and other groups (Gould et al., 2006; Salome et al., 2010). In the discussion, they argue that this might be due to the involvement of these clock factors in light input. In would strengthen their point if the temperature compensation of phytochrome mutants was also presented. In addition, they need to show in the model, what is the effect of introducing temperature regulation of the non-light related parameters and compare it with changes in only the light related parameters.

The selection of which light-dependent parameters were modified is also not well explained. Only some of the light dependent parameters were modified to include temperature dependency (7 out of 12; Sup. Table 3). However, when trying to find parameters to fit the cry1cry2 model, the authors mention attempting to change the parameter of PRR9 protein degradation. This parameter was not temperature dependent in the first analysis. Therefore, the selection of temperature-dependent parameters seems rather arbitrary. Furthermore, on page 9 the authors argue that only a subset of the parameters for which temperature dependence was introduced had substantial control coefficients for period length. It is unclear what they called "substantial" control coefficients, since all the parameters modified (a total of 7) had values > +/- 2 (Sup. Table 3).

Interestingly, the LHY protein levels increase at elevated temperatures although LHY RNA levels

do not change, which fits the model predictions. The model shows that this increase is due to an increased translation rate caused by the introduction of temperature dependency of light-related parameters. In the plant, however, LHY protein accumulation might also be caused by a reduction in degradation rate. In order to show that the accumulation corresponds to the hypothesized increase in translation rate, potential changes in LHY protein stability and/or LHY translation should be tested. It would also strengthen the manuscript if the LHY protein levels at 12C and 27C were analyzed in the cry mutants.

More details on the modeling sections would be useful to the reader. For example, the model equations and a graphical representation of the model should be presented in the supplemental material. The equations used to constrain the model should also be shown; this will clarify the criteria used and help the reproducibility of the model by other groups. For example, when fitting the temperature dependent model to the different temperatures, were only the period lengths taken into account or also the phase of expression of the different clock genes, as was the case in the original model? Were the new temperature-dependent parameters changed by hand?

Other comments/questions:

It would be helpful if the authors discuss why the cry1cry2 double mutant displays only weak circadian phenotypes at 12C and 17C although is has significant changes in clock gene expression (Figure 2).

When calculating the period length, which was the RAE cut off used to define a plant as arrhythmic?

What were the red and blue light intensities under which the experiments were done?

Figure 2: Are the expression levels just normalized to UBQ10 internal standard, of are they normalized within each experiment as well?

Main text: check separation between literature reference and text, in several cases a space is missing.

Supplemental Information:
p.4 last paragraph: there is negative sign missing in the Arrhenius equation.
p. 6 "Light also effects..." should be "affects"

Reviewer #3 (Remarks to the Author):

That light and temperature are Zeitgebers for circadian rhythms is well established. It has also been known for quite some time that temperature and light may act similarly. While light and temperature have often been considered as independent environmental variables when trying to explain light and temperature effects, in this manuscript, Gould et al. take us one step further, by using a systems approach and showing that light and temperature play a converging role in temperature compensation, one of the defining clock properties of circadian rhythms.

As I see it, this is the first (systems-based) approach showing that light and temperature have a converging role in the plant biological clock and that temperature compensation is due to a network balance. This conclusion is supported by a large set of experimental data and by model computations presented by the authors.

I have a few minor remarks that the authors may consider in a revised version:

i) on page 3 the authors refer to the balance equation by writing W = c1d1+...+cndn. For clarity, the authors may mention that W is a measure which describes how the period depends on temperature and then refer to the Supplementary material where a more explicit form of the balancing equation is shown. For readers not familiar with metabolic control theory and how control coefficients are defined the form of the W, ci's and di's may not be obvious.

ii) On the bottom of page 3 the authors discuss how "global" temperature compensation in the

Neurospora circadian clock can be explained by two isoforms of the clock protein FRQ. However, temperature compensation is also observed when only one of the isoforms is present, but in this case temperature compensation is observed in a smaller temperature range. A slightly alternative view how to explain temperature compensation in the case with an "averaged FRQ protein" is due to a balance between FRQ degradation and FRQ synthesis processes as has been done in PNAS 2005, 102, 17681-17686. This could be briefly mentioned as a complementary remark.

iii) To make the access to the model (Pokhilko et al. 2010) easier the authors may consider to describe (repeat) explicitly the equations of the model in the supplementary material or mention the database if the model has been submitted to one.

In summary, this manuscript shows novel findings how temperature compensation is achieved in the plant circadian clock and I recommend publication in MSB.

Reviewer #4 (Remarks to the Author):

In this manuscript, Gould et al. investigate the mechanism of temperature compensation in the Arabidopsis circadian clock by looking at interactions between light quality and temperature on oscillations of clock outputs. They observe that temperature compensation requires blue light at high (27∫C) temperature and that this depends on functional cryptochromes, a class of blue light receptors. The authors develop a model to show that light and temperature converge on common targets within the clock, and attempt to test this model by examining predictions that temperature compensation is achieved through alteration of LHY protein abundance to affect PRR9 transcript expression.
This is a well-written manuscript which adds to our understanding of temperature compensation in the Arabidopsis circadian network with respect to the involvement of blue light signaling. However, a number of issues, some major, need to be addressed before publication.
General comments
1. Although it is clear that CRY1 and CRY2 are critical for temperature compensation at 27∫C, they appear not to contribute at 12∫C since there is no difference between rhythms of WT and cry1cry2 at this temperature. Furthermore, the period of WT appears significantly shorter under red light or red+blue light at low temperatures and rhythms under blue light at 12∫C, particularly of CAB2:LUC, approach arrhythmia. This suggests that red light signalling might be more important at low temperatures. Therefore, it seems appropriate that the authors should have investigated the effect of red light receptors (phytochromes) or ZTL, another putative blue light receptor, on temperature compensation.
2. I am not entirely satisfied with the model validation presented in this manuscript. Testing of the predicted behavior of LHY protein and PRR9 transcript under the model does not indicate whether this is a mechanism (as suggested p 14 para 2) or a response of temperature compensation. Particular as the model is highly constrained to reproduce the experimental data. A better test of this as a mechanistic hypothesis would be to assess temperature compensation in lhy and prr9 mutants. If the model is correct, I expect that lhy and prr9 should be impaired in temperature compensation. Furthermore, the suggestion that "temperature compensation is primarily effected through temperature-dependent modulation of light input pathways into the morning loop of the clock" is misleading. The authors have not experimentally shown that there is not an involvement of the evening loop either by analysis of mutant phenotypes or extensive analysis of, for example, protein abundance of evening loop components.
3. The authors must place this work within the context of previous experimental work on analysis of clock mutants on temperature compensation of the clock (Gould et al., 2006; Salome et al., 2010). For example, it seems to have been largely ignored in this manuscript that GI, a component of the evening loop, has previously been suggested to be involved in temperature compensation. This is surprising since this was the major finding in the previous manuscript by these authors on this topic (Gould et al., 2006). Furthermore, the phenotypes reported for various mutants in these publications might support the hypothesis put forward under the model in this manuscript. For example, Salome et al reported 'overcompensation' in prr7prr9 mutants in addition to the defects on temperature entrainment in these mutants.
The major concern is that the findings concerning LHY appear to be at odds with the authors own previous findings of Gould et al., 2006. in the current manuscript Page 11 "Our experimental data matched the prediction, as peak LHY levels were 2.7-fold higher at 27{degree sign}C than at

12{degree sign}C (Figure 3B and C)." This is the complete opposite to the finding of Gould et al 2006 "in which increases (from 17 to 27{degree sign}C), (caused) decrease for LHY" . In the current manuscript it is argued that LHY mRNA increases with temperature yet in their previous manuscript it is argued LHY decreases with temperature. This apparent discrepancy and the authors failure to address the discrepancy leave the reader uncertain about the reproducibility of the results and therefore I wonder if the interpretations are valid.
4. Why were the temperature induced changes in CCA1/LHY, GI and TOC1 levels reported previously by the authors (Gould et al., 2006) not included in the model? In the previous manuscript the authors claimed that introducing these changes in to the Lock et al., (2005) model of the circadian clock was sufficient to recapitulate the experimental temperature compensation phenotypes. If this is the case, why is it necessarry to include also the effects of blue light signaling? Do the conclusions of the current manuscript still apply if the changes in clock transcript abundance are included in the model? Or is it the authors are no longer secure in the results described in Gould et al., 2006 because of the apparent difference in the data from the current manuscript?
Does the recently finding that TOC1 is repressive to CCA1 alter the conclusions of the manuscript?
Specific comments
5. p 8 line 3. This sentence is misleading. There appears to be no difference between cry mutants and WT at 12∫C.
6. p 8 paragraph 2. The final sentence is misleading. CRY proteins could be switching between active/inactive forms in a temperature-dependent manner. While is it true that this is downstream of CRY protein accumulation, I feel the implication of this paragraph by the authors is to suggest that temperature is not having an effect on CRY activity directly, which is not necessarily true.
7. p 9 line 6. If LHY and CCA1 transcript levels at or below WT trough levels is the criteria for arrhythmia, then these transcripts are arrhythmic at all temperature in cry1cry2. This sentence should be revised.
8. Discussion, second paragraph and fifth paragraph. It is not clear to me that either a network-balancing model or a temperature-(in)sensitive component model is particularly supported over the other. It is very difficult to distinguish the two. The nature of the circadian network is such that an effect on a single component will have broad effects on the network. Furthermore, the possibility that the temperature effects are not mediated through modulation of CRY activity, for example, has not been excluded.
9. Were the fluence rates of light for each light quality the same? These should be given in the methods.
10. Figure 1. The data-points for cry1 in most of the panels in Fig 1B are not visible. Is it possible for this to be improved for clarity?
11. Figure 2.
a. What does an mRNA level of "1" mean? It clearly cannot mean transcript levels equal to UBQ10, since this reference transcript is expressed at higher levels than CCA1, LHY and TOC1 (as previously reported by these authors and others). If the data has been normalized to a data-point, this should be described in the legend and/or methods. Also, the y-axis should be labeled as 'Relative mRNA levels". However, it is this reviewer's preference that the true transcript/UBQ10 value be given.
b. The model seems to poorly predict amplitude and shape of the oscillation in cry1cry2 at 12∫C and 17∫C, particularly of LHY and CCA1. Is this an artifact of the normalization of the transcript data, or does this actually provide insight into potential inadequacies in the model?
12. Figure 3. Why do the authors focus on LHY (here and elsewhere) rather than both LHY and CCA1 when these are not distinguished in the Pokhilko 2010 (and preceding) models? This should be explicitly explained somewhere in the manuscript.

Re-submission 27 June 2012

I would like to thank you very much for the time and effort that has gone into reviewing this paper. We are pleased that the reviewers, and you as editor, recognised the interest of this work and that upon addressing the comments raised by the reviewers you would reconsider this paper for publication in Molecular Systems Biology. However, we did have one particular concern, that some of the reviewers suggested looking at single molecules or processes, such as cryptochrome kinase activity, which misses the point of the paper. While it is entirely feasible that the kinase activity could affect temperature compensation, it must do so by controlling biochemical processes in the

clock gene network that we model here. Its effectiveness would then depend upon the size of the corresponding control coefficients, which we study. However, there are many other enzymic reactions that could affect temperature compensation within the network. Individually knocking genes out and characterising their temperature compensation phenotype does little to help the ultimate goal of understanding the mechanism of temperature compensation of the entire network. The point of our approach is to consider the whole system rather than a single component. Our work has surprisingly identified that by adding temperature functions to just a subset of light signaling components within the network, we can match a complex experimental data set. These results, together with our conceptual arguments, provides evidence for a common input of light and temperature into the clock.

Below, we address all the concerns of the reviewers and we believe the manuscript is now strengthened, improved and suitable for publication.

Editor's specific points

- *"analysis of phytochrome mutants would be essential".*
  We have added an analysis of the temperature dependent effects of phytochrome mutants on the circadian clock. The experiments are identical to those described for the cryptochrome mutants and our statistical analysis now incorporates the phytochrome data. We have included this analysis in supplementary figure 2, table 1 and supplementary tables 1 and 2. The analysis is described on pages 7 and 8.

  The subtle effects of the phytochrome mutants match previous results (Devlin and Kay 2000) and justify our focus on cryptochrome.

- *"the reviewers indicated that the temperature compensation model presented here required more thorough and direct validation."*

  In our previous submission, the model predicted that LHY protein levels increased at high temperature, and this prediction was validated. Furthermore, the model was able to qualitatively match results from the *cry1* and *cry1 cry2* double mutant. We have now extended this validation:

  1. Our simulations predicted that LHY protein levels would be arrhythmic and low at 27°C in the *cry1 cry2* double mutant. We have validated this prediction (see new figure 3).

  2. We have extended our analysis to include a simulation and validation of the temperature dependent phenotypes of the *cca1 lhy* and the *prr7 prr9* double mutants (new supplementary figure 6).

We also place the work more fully in the context of previous works (Gould *et al,* 2006; Salome *et al,* 2010) (reviewer #4, points 3 and 4).
I hope you find our response complete and satisfactory.

Specific comments :

Reviewer #1.
"1. I know it sounds good, but you did not have 90,000 useable data points as mentioned on page 6. You cannot do anything with the single LUC activity data of single time-points, so I would like this statement deleted. Just say how many period values you generated (1900 or so, as mentioned on top of page 7)"

We now include more data but have deleted this statement, this is however, one of the largest circadian data sets analysed and as such requires a statistical modelling approach to analyse our data. It was for this last point we wanted to put this statement in to emphasise the need for a statistical model. We have, as suggested by the reviewer, added the number of periods analysed, now 2748.

"2. I imagine only half of the data collected on photoreceptor mutants is being shown here: why not report the data on phy mutants (phyA, phyB and phyA phyB) that the authors must have collected?"

phy mutant data are now included, as noted above in our response to the editor. The distinct response of the circadian clock in RL compared to BL, even in WT plants, argue for a focus on one or the other.

"3. in the Devlin and Kay article (Plant Cell 2000. 12: 2499) a cry1cry2 double mutant had a long period for fluence rates of red light ~40 µmol/sec/m2 and below. I realize this was at 22ºC, but Figure 1C in this manuscript shows very little effect of red light in the cry1cry2 double mutant. Yet, on page 7, 5 lines before the end of the page, the authors state that their results agree with Devlin and Kay"

"4. What is the fluence rate being used here, for red and blue light, alone and combined? This might in part explain the above discrepancy, if red light fluence rates were above 50 µmol/sec/m2 (which would be quite high). This could also explain why cry2-1 has almost no effect on period: fluence is too high"

The light levels we use have been added to the materials and methods section.
"The illumination within the cabinet was provided by either a RL LED array (30 µmol·$m^{-2}s^{-1}$), a BL LED array (30 µmol·$m^{-2}s^{-1}$) or a R/BL mixed array (20 µmol·$m^{-2}s^{-1}$ RL, 20 µmol·$m^{-2}s^{-1}$ BL)."
At the fluence rate used in our analysis, the *cry1 cry2* double mutant had no phenotype under RL, consistent with published data from Devlin and Kay 2000. They showed a period change specific to lower fluence rates.

"5. It is a shame not to have included data points at 22ºC. This generates a big gap in data, from 17ºC to 27ºC, and prevents direct comparison with others' results"

Our conclusions do not depend on comparisons to previous data that are specific for 22°C. Moreover, increasing amounts of data are being gathered by other laboratories at 17°C and 27°C, allowing direct comparison to our data.

"6. About the analysis: in my mind, 96 hours is too short. 120 hours would have been preferable, especially because I would start the analysis only on ZT12 on the first day in LL for CAB2, and even later for CCR2. One of the reasons RAEs are so high at 27ºC for CCR2 in blue light is likely to because of the first peak in LL, which has a higher amplitude. RAEs are informative in describing strength of rhythm, but only when amplitude does not vary too much. RAE only measure the fit to the sine wave fitted by FFT-NLLS. I think FFT-NLLS latches on to this first peak and fits the sine wave to it, to the detriment of the remaining cycles"

The analysis was consistent for all data, starting at ZT12 (please see response to reviewer 2 point 7). The reviewer is correct that the FFT-NLLS method assumes stationarity in the data, like other widely used rhythm analysis methods. The principal conclusion that we draw from the 27°C data is from the comparison of the WT to the *cry1 cry2* double mutant. Rapid loss of rhythmic amplitude is the key phenotype in the mutant. The RAE measure appropriately reflects this phenotype, and distinguishes it from WT, for exactly the reasons that the reviewer outlines.

"8. the symbols in Figure 1 are too big and too numerous, so one cannot tell them apart. Since the authors make no comments about the direction of RAEs vs period length, I would redo the graphs as box or dot plots for RAE data only; the period data are already shown in Figure 1C"

We have altered the symbols as suggested by the referee (see new figure 1).

"9. It would be very useful to see the mean traces before normalization, for a representative transgenic line.

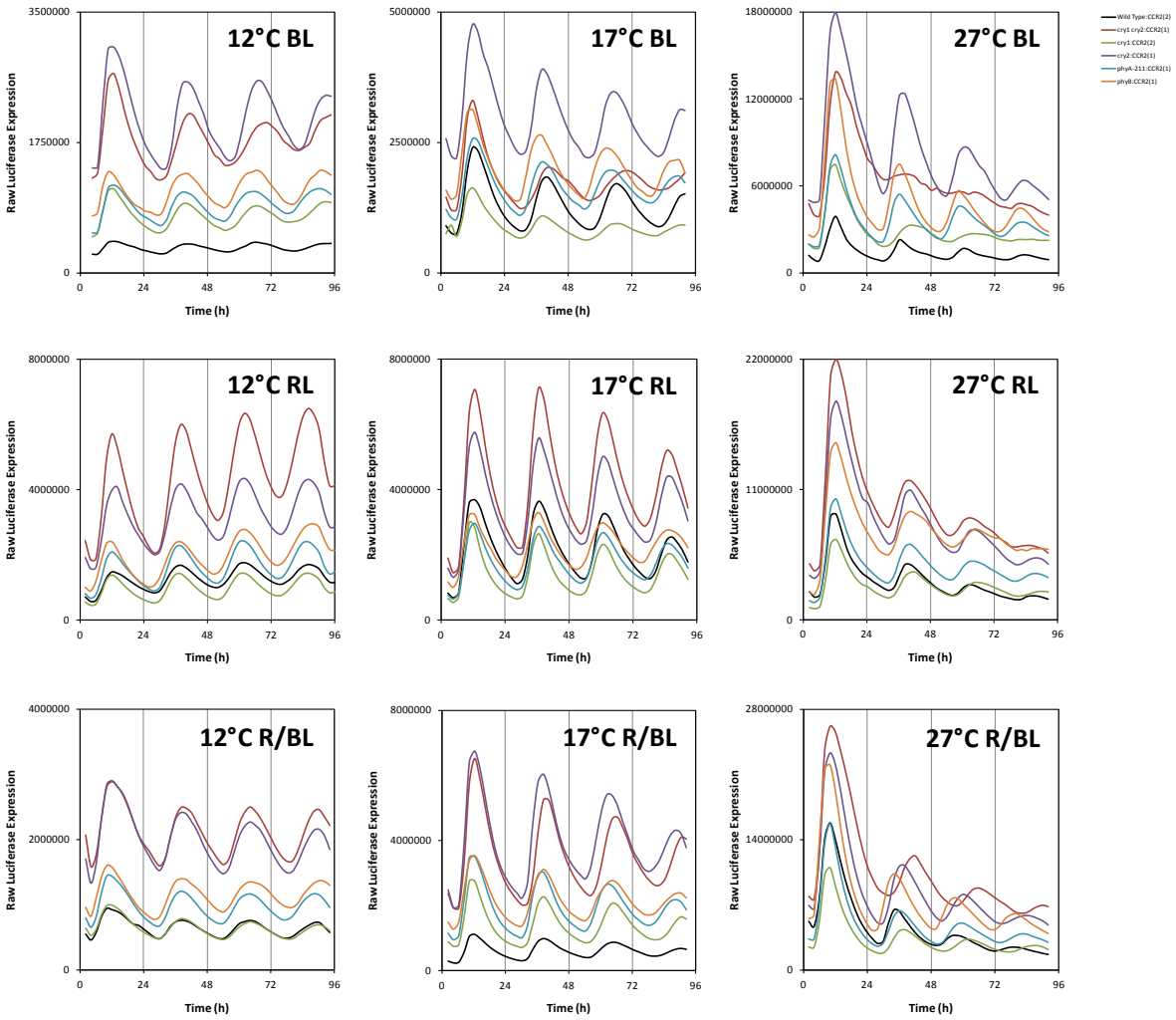


Figure. Non-normalised averaged data for representative lines across 12, 17 and 27°C in RL, BL and R/BL conditions.
Transgenic Col-0 WT, *cry1, cry2, cry1 cry2* double, *phyA* and *phyB* mutant seedlings carrying the *CCR2:LUC* reporter gene were entrained under 12L:12D cycles for 7 d, transferred at ZT 0 to 12, 17, or 27°C and imaged under constant BL, RL or R/BL. Each plot shows a representative mean trace of a single line of raw non-normalised. The data was generated from groups of seedlings (n=10-20) of each mutant studied versus WT.

10. what were the traces normalized to? Mean LUC activity over the whole time-course for each seedling?
This sentence was expanded in the materials and methods:
"Luminescence levels of groups of seedlings (10-20 seedlings) were analysed by low-light imaging as described previously (Gould et al., 2006), normalising levels to the mean LUC activity over the whole time-course for each group."

11. I would also like to see traces for cry1cry2 at 27ºC in blue light, for the arrhythmic set and for the rhythmic set"

We agree with the reviewer that it is important to for readers to have access to raw data. Upon publication we will make all data available via Biodare, as stated in the Methods section. Biodare is a web based database and analysis platform for circadian research. However, we have also replotted the requested data set (see below) as asked for by the reviewer.

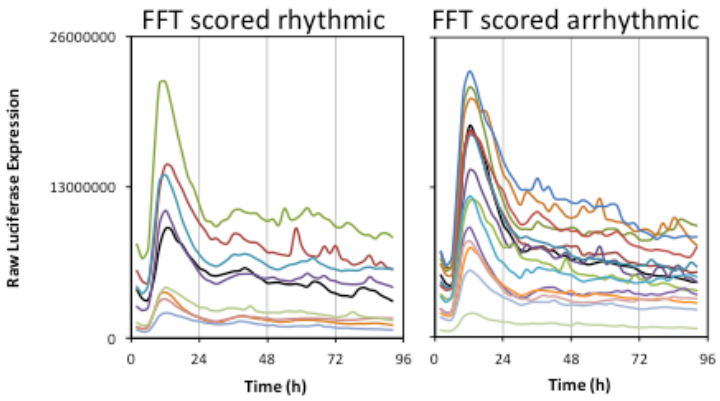


Figure. Non-normalised luminescence for individual groups of *cry1 cry2* mutant seedlings carrying the *CCR2:LUC* reporter under BL at 27°C. The data has been split with those giving rhythmic period estimates on the left and those arrhythmic estimates on the right.

"12. 38% to 44% of cry1cry2 seedlings are still rhythmic in blue light at 27ºC, and display a normal period length. This does not qualify as strongly arrhythmic, unlike an elf3 mutant for example. Can the model explain the remaining rhythmicity?"

This is an excellent point and we have added a discussion of how our model predicts a population of rhythmic and arrhythmic individuals.

"One observation from our data is that the *cry1 cry2* double mutant was not completely arrhythmic, in fact, 38% of the samples returned a rhythmic value for their expression of *CCR2*. The FFT-NLLS analysis method identifies a minority of near-circadian periods with high RAE in the fluctuating expression patterns of all arrhythmic mutants, such as *elf3* (Hicks, *et al.* 1996). However, the remaining rhythms could also be explained from our model. In BL the *crr1 cry2* double mutant at 12, 17 and 27°C is close to a Hopf bifurcation (see discussion in supplementary material). This means that by small alterations in some of the parameters one can change the damped oscillations to a sustained oscillation or *vice versa*. At 12°C the data and model suggest that there is a relatively low amplitude limit cycle and therefore sustained oscillations. At 27°C the model suggests that the system is so close to a Hopf bifurcation that one should expect to see a mixture of behaviours for the experimental system. We believe that this is why we observe that at 27°C approximately 40% of *cry1 cry2* mutant samples are still rhythmic and with period lengths clustered around the WT, rather than a completely arrhythmic phenotype."

Please note also our response to reviewer 2 point 7. Hicks *et al*. 1996 used an adaptive threshold in the RAE value to eliminate some near-circadian periods from the *elf3* mutant, and other authors have used an arbitrary RAE threshold for this purpose, whereas we report all periods 15-35h, as described in the Methods section.

"13. the last sentence of the first paragraph on page 8 bothers me. For one, it is too early to make this suggestion based on the data thus far. Second, it skirts around an issue that admittedly took me a while to formulate. The Somers and Devlin articles on circadian entrainment by photoreceptors looked at free-running period under a range of fluences at a single temperature. The authors here (and others before) measured free-running period at a single fluence and over a range of temperatures. In both cases, seedlings were first entrained to LD cycles, then released in constant conditions. In both assays, the seedlings are thus grown under the same conditions.
When does one decide that a change in period reflects a problem with entrainment of the clock vs compensation of the clock? This is rather important since the authors claim that compensation goes through photoreceptors, and thus circadian entrainment will be affected"

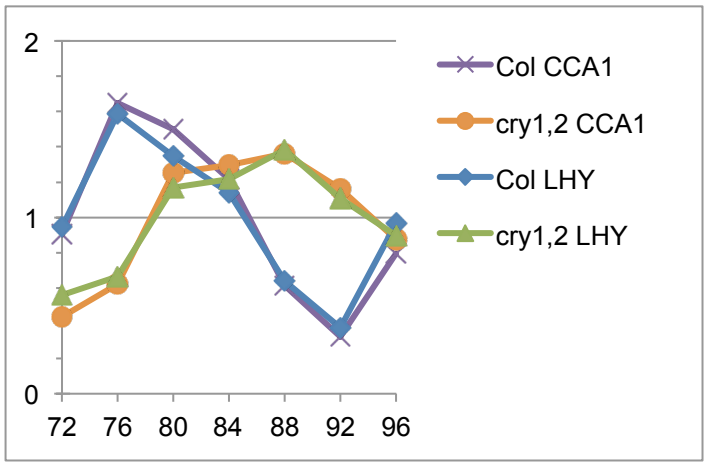
We do not understand why the reviewer brings in entrainment here, though their point is interesting. However, the sentence he/she highlights concerns the effects of temperature and light quality on period. We do not address entrainment by light or by temperature in this paper.

"*14.the authors used LUC reporters for output genes, and they might therefore not reflect what the clock is really doing. Another reporter, like CCA1:LUC or TOC1:LUC or PRR9:LUC, all used in the 2010 Pokhilko article, would be a lot better. Data from Figure 2 could be used to infer the behavior of Col-0 at the various temperatures in blue light, but not for cry1cry2*"

The referee's general point is well taken, therefore in this paper we used qRT-PCR to quantify the effects on central clock components directly. However, the stable period of a coupled system is the same for all components, so one can use any part of the system as a marker. *CCR2* and *CAB2* LUC are established clock markers that have been used in many publications.

"15. in Figure 2, I would argue that all clock genes are arrhythmic in cry1cry2 at all temperatures in the experimental dataset, but not in the model. In fact, the model appears to perform poorly at all temperatures when predicting clock gene expression in the double mutant"

We agree, as noted below, that the clock gene profiles are altered in the double mutant at all temperatures (and please note our response to point 12). The fact that the destructive RNA analysis necessitates testing different individuals at each time point can complicate the analysis of clock mutants with weak rhythms. However, the reviewer may be misled by the low expression levels of some of the clock genes, which make their continued rhythmicity harder to see in Figure 2. For example, the plot at right shows the same data as Figure 2 for *LHY* and *CCA1* expression levels at 12C, normalised to their respective means. The rhythmic amplitude of these clock components is still strong in the double mutant despite the lower average levels of expression.



To clarify this point, we now quantify this change in the results section: "In contrast to WT, the loss of cry photoreceptors profoundly altered gene expression levels. The *cry1 cry2* double mutant showed *LHY* and *CCA1* mRNA levels at 12°C peaking at trough WT expression levels, with lower fold amplitude (3.2-fold, compared to WT 4.3-fold)."

While no model is likely to be perfect, the fact that this model captures much of the qualitative behaviour of the *cry1 cry2* mutant is very encouraging. This is discussed in the supplementary information:

"This model of the *cry1 cry2* double mutant showed the loss of rhythmicity at 27ºC, and showed a good match in period profile at low temperature (28.45h at 12ºC), but predicted a lower period at 17ºC than observed (25.1h at 17ºC) (Supplementary Figure 5). Most of the genes relative mRNA levels matched the experimental data for the double mutant compared to the WT (Figure 2). For *GI* mRNA, the *cry1 cry2* double mutant levels were consistently too low compared to the data, which could reflect the limited constraints available during the construction of the original Pokhilko 2010 model, as discussed (Pokhilko et al, 2010). Thus, with this possible exception, the model circuit proved sufficient to match both the period profiles and the RNA time-series data."

The model also successfully predicted low and arrhythmic levels of LHY protein at 27ºC in the double mutant (figure 3).

"16. all models to date have had TOC1 as a positive regulator of CCA1/LHY expression. However, the latest data from the Kay lab shows that TOC1 is actually a transcriptional repressor. I know the Kay article was published online on February 6, so about one week after this manuscript was submitted. However, one of the co-authors (Karen Halliday) attended the 2011 Photobiology meeting in China, during which Steve presented these results. Given how badly the model seems to

be at predicting clock gene expression in cry1cry2, wouldn't it be better to try and figure out why none of the models anticipated the repressor role of TOC1, even though all models were presumably based on experimental data?"

The reviewer asks *"wouldn't it be better to try and figure out why none of the models anticipated the repressor role of TOC1, even though all models were presumably based on experimental data?"* Part of the Millar lab's approach has been to test whether the hypotheses proposed in the literature were consistent, when formally expressed in mathematical models. A key advantage of models is to make explicit any assumptions that are required to do so. Since Locke et al., Mol. Syst. Biol. 2005, the Millar lab's papers have argued that TOC1 was likely to be an activator of *LHY* and *CCA1*, *only if* another component (X, or TOC1mod) existed to create the required delay between TOC1 expression and *LHY* and *CCA1* transcription. This was clearly more parsimonious than the alternative proposal, which would require both a change in the sign of TOC1 function and another component to create an oscillator. More complex models can always be proposed.

The reviewer implies that only modelling could have suggested an alternative hypothesis. Though this is complimentary to modellers, experimentalists are also expected to interpret their results. The Kay and Mas labs clearly did, and consequently tested the alternative hypothesis.

"17. the authors state in this manuscript that the parameters in their 2010 model (at 22ºC in combined red and blue light) are similar to the experimental data they collected here in blue light at 27ºC. This likely is due to the absence of data in this manuscript at 22ºC. Again, it would have been very nice to collect data at 22ºC, for no other reason than to extend the 2010 model. I think this contributes to the poor performance of the new model"

The model actually performs well. We provide an argument for this in the main paper and expand this in the supplementary information.

"The period of our recent model (Pokhilko et al., 2010) simulated in constant light was similar to the profiles measured at 27°C under BL in WT plants, so we used the published parameter set (Pokhilko et al., 2010) to represent this condition"

"Our original 27°C model took all its parameter values from the Pokhilko et al. 2010 model (modelled on 22°C white light experimental data), because this model has a free-running period of 24.5h, matching the period profile of the BL data at 27°C."

"18. are LHY protein levels changing in the cry1cry2 double mutant between 17ºC and 27ºC? And what happens at 22ºC in both Col-0 and the double mutant?"
We agree with the reviewer this is sensible. Our simulation predicts that at 27°C LHY protein levels will be low and arrhythmic in the *cry1 cry2* double mutant. We have now tested this prediction experimentally and added the results to new figure 3.

"19. Cryptochromes are kinases, and therefore enzymes. Couldn't their kinase activity be temperature-dependent?

While it is entirely feasible that the kinase activity could affect temperature compensation, it must do so by controlling biochemical processes in the clock gene network that we model here, presumably through the parameters that represent the light input pathway. Therefore the temperature dependence of cry activity is covered in the present context by making these parameters temperature dependent. The extent to which any such parameter affects temperature compensation depends upon the size of the corresponding control coefficients, as we explain.

20. Indeed, crys are not known to interact with any of the clock proteins. This only means that they never showed up in a general 2-hybrid screen. But the TOC1/ZTL interaction only came from a directed 2-hybrid, so it would be worth looking into this. cry1 autoactivates, but it can still be used as prey against the other clock proteins. "

The identification of additional protein-protein interactions, while interesting, is beyond the scope of the present manuscript.

"21. I have never seen this discussed anywhere before, but luciferase is also an enzyme, so its activity will change with temperature. This should be taken into account when comparing amplitudes between different temperature treatments"

At each temperature we compare WT to mutant. Absolute luminescence levels (and thus absolute amplitude) is likely to change. However, the circadian period of expression is unlikely to be affected (see response to point 14 above) nor is the amplitude measure that we use (in common with much of the field), which is the relative amplitude: peak level divided by trough level. All statements in the paper about changes in levels rely on qRT-PCR and western blots, not on luciferase.

"22. the fourth sentence of the introduction should say "unusually temperature-independent", not "unusually temperature-dependent""

We realise that this sentence was confusing; we have now written:

"evolved in order to provide unusually temperature-dependent or temperature insensitive regulation"

"23. on page 12, first paragraph of discussion: although cry1cry2 mutants remain rhythmic in blue light, this is not the case for the phy quintuple mutant in red light"

The reviewer is correct that the *phy* quintuple mutant appears arrhythmic in red light for rhythms in leaf movement. The quintuple mutant is still rhythmic in WL, albeit with a short period. We have altered the last sentence in the discussion to make our statement more precise.

"Either red or blue photoreceptor pathways alone are sufficient to support similar rhythms (Figure 1), and previously it had been shown that plants lacking both cryptochromes (Devlin and Kay, 2000) or all phytochromes retained circadian rhythms in constant WL (Strasser et al., 2010)."

"24. arrhythmia should be changed to arrhythmicity"

As both are used within the field, we would be happy to change this at the editor's discretion.

"25. all seedlings were first entrained with LD cycles comprising of white light. When relative to ZT0 or ZT12 were the seedlings transferred to blue, or red, or blue + red?"

We have now added the statement
"Seedlings were transferred to constant conditions at ZT0."

Reviewer #2
1. "It would strengthen their point if the temperature compensation of phytochrome mutants was also presented"

We have added the data requested, please see the response to the editor's specific comments above.

2. "In addition, they need to show in the model, what is the effect of introducing temperature regulation of the non-light related parameters and compare it with changes in only the light related parameters"

In the paper, we do not state that the light parameters are the only possible candidates for temperature sensitive parameters. It is most likely that all parameters within the model are temperature sensitive. Our statement is, that by considering a subset of parameters, namely, the light-related parameters, we can already model and explain a great deal of our data. Clearly, including a wider set of non-light-related parameters could help refine the fit, but this is not what we have set out to show.

3. "The selection of which light-dependent parameters were modified is also not well explained. Only some of the light dependent parameters were modified to include temperature dependency (7

out of 12; Sup. Table 3). However, when trying to find parameters to fit the cry1cry2 model, the authors mention attempting to change the parameter of PRR9 protein degradation. This parameter was not temperature dependent in the first analysis. Therefore, the selection of temperature-dependent parameters seems rather arbitrary. Furthermore, on page 9 the authors argue that only a subset of the parameters for which temperature dependence was introduced had substantial control coefficients for period length. It is unclear what they called "substantial" control coefficients, since all the parameters modified (a total of 7) had values > +/- 2 (Sup. Table 3)"

The selection of temperature-dependent parameters is not arbitrary. We restricted ourselves to parameters of the light input pathways in order to test a key hypothesis as is explained clearly. Of these we only model as temperature-dependent those with a significant control coefficient. As we explained early on in the paper because of their small control coefficients adding the others would not have a significant effect and would unnecessarily complicate the model. We have added a sentence to clarify this:
"Our selection criteria is that the parameter has to have at least one sensitivity coefficient above 20% of the largest sensitivity coefficient at all temperatures (LHY mRNA degradation at 27ºC)."
When trying to model the *cry1 cry2* mutant we found it necessary to bring in consideration of PRR9 degradation. This sort of approach enables one to predict what are likely to be the key mechanisms.

4."*It would also strengthen the manuscript if the LHY protein levels at 12C and 27C were analyzed in the cry mutants*"

We have added a simulation of LHY protein levels in the *cry1 cry2* double mutant and tested the prediction of low LHY and arrhythmic protein levels at 27°C experimentally see figure 3.

5. "For example, when fitting the temperature dependent model to the different temperatures, were only the period lengths taken into account or also the phase of expression of the different clock genes, as was the case in the original model? Were the new temperature-dependent parameters changed by hand?"

We have now included a list in the supplementary information that outlines all modeling constraints, ranked by order of importance. These constraints include, but are not limited, to period lengths and relative phases of expression. Only relative phases of expression are included because absolute phase of expression is not meaningful unless one is considering entrained systems (which we are not doing here) and hence is not included.

"Our list of modeling constraints ranked in order of decreasing importance is:

1. Period profile in WT plants at 12°C, 17°C and 27°C. Relative levels of clock gene mRNA in WT plants across 12°C, 17°C and 27°C (more precisely, *PRR9* mRNA level triples from 12°C to 27°C, *TOC1* level doubles, and all other mRNA levels stay constant across all three temperatures). Phase differences among clock gene rhythms in WT plants at each temperature (Figure 2).

2. Period profile in *cry1cry2* mutants. Low mRNA levels in the *cry1cry2* double mutant at each temperature compared to WT mRNA levels.

3. Period profile in *cry1* single mutants."

Changes to temperature-dependent parameters were guided by the information from balance equations and from sensitivity coefficients.

6. "It would be helpful if the authors discuss why the cry1cry2 double mutant displays only weak circadian phenotypes at 12C and 17C although is has significant changes in clock gene expression (Figure 2)"

This is a very good point and we have added this section to the discussion:

"One surprising observation was that clear circadian rhythms in the clock outputs *CCR2* and *CAB2* were observed at 12°C and 17°C under BL in the *cry1 cry2* double mutant (Figure 1). The molecular rhythms of clock component RNAs were more strongly affected (Figure 2), though *LHY* and *CCA1* retained rhythmicity with only slightly lower fold amplitude than in WT. This is not without precedent, as clear oscillations with near-normal periods were observed in constant far-red light, despite major shifts in the levels of clock gene mRNAs (Wenden et al., 2011). Complex output pathways that include non-linear feedback structures can, in general, filter and potentially amplify rhythmic signals, and these structures are present in the control of *CCR2* and *CAB2*. The *EPR1* transcription factor, for example, is rhythmically expressed, controls *CAB* expression and shows auto-regulation (Kuno *et al*, 2003). The CCR2 RNA-binding protein, likewise, contributes to the control of *CCR2* mRNA levels (Schöning *et al*, 2008)."

7. "When calculating the period length, which was the RAE cut off used to define a plant as arrhythmic?"

There is no generally applicable RAE threshold for arrhythmia, because the absolute values of RAE scores in WT data depend upon the particular rhythmic output and the amount of data analysed. The first paper on this approach used an adaptive threshold based on the WT RAE distribution (Hicks et al., 1996). Here, no RAE cut off was used, as we now clarify in the materials and methods:

"To avoid light-driven responses and ensure consistency across all experiments, 96h of data were analysed for each plant starting from ZT12, retaining any rhythm estimates with 15-35h period. Arrhythmia was defined as a failure of FFT-NLLS to identify any period in this range."

8. "What were the red and blue light intensities under which the experiments were done?"

This has been added to the materials and methods, please see reviewer 1 point 4.

"Figure 2: Are the expression levels just normalized to UBQ10 internal standard, of are they normalized within each experiment as well?"

This is now clarified in the materials and methods section. Levels of RNA for each transcript were quantified relative to experimental calibration curves that measure PCR efficiency for each primer set. The calibration is relative, not absolute. The level of each clock RNA is then normalised to a *UBQ* reference RNA, allowing comparison between samples and across replicate experiments.

"The Relative RNA level for each sample was calculated:
Relative mRNA = relative measure of candidate mRNA/ relative measure of *UBQ* mRNA."

Reviewer #3

"*i) on page 3 the authors refer to the balance equation by writing W = c1d1+...+cndn. For clarity, the authors may mention that W is a measure which describes how the period depends on temperature and then refer to the Supplementary material where a more explicit form of the balancing equation is shown. For readers not familiar with metabolic control theory and how control coefficients are defined the form of the W, ci's and di's may not be obvious."*

The text been adjusted as advised by the reviewer and definitions expanded.

"This applies to any differential equation model of the clock. It asserts that the overall effect of temperature *T* on period is a sum of terms, one for each of the temperature dependent parameters k_j, and that each of these terms is a product of two quantities: a measure of the way in which the period depends upon the parameter (the parameter's control coefficient C_j) and a measure d_j of the way the parameter depends upon *T*. We can write this $W = \sum_j C_j\, d_j = C_1 d_1 + \cdots + C_s d_s$ where *W* measures the change in period for a unit change in temperature. Thus, for temperature compensation a necessary and sufficient condition is $W \sim 0$. In the first hypothesis above, specific molecular

structures have evolved so that some subset of the d_js have a significant functional dependence upon *T*, which makes the principal contribution to ensure that $W \sim 0$ (for a more extensive discussion see the supplementary material)."

"ii) On the bottom of page 3 the authors discuss how "global" temperature compensation in the Neurospora circadian clock can be explained by two isoforms of the clock protein FRQ. However, temperature compensation is also observed when only one of the isoforms is present, but in this case temperature compensation is observed in a smaller temperature range. A slightly alternative view how to explain temperature compensation in the case with an "averaged FRQ protein" is due to a balance between FRQ degradation and FRQ synthesis processes as has been done in PNAS 2005, 102, 17681-17686. This could be briefly mentioned as a complementary remark."

We have added this alternative view and suggested reference to the introduction.

"An alternative approach to temperature compensation in Neurospora proposes that it is due to a balance between FRQ degradation and FRQ synthesis. This is an example of such network balancing (Ruoff *et al*, 2005)."

"iii) To make the access to the model (Pokhilko et al. 2010) easier the authors may consider to describe (repeat) explicitly the equations of the model in the supplementary material or mention the database if the model has been submitted to one."

We have added details as to where the model can be downloaded in the supplementary material.

"The P2010 model is accessible from the Biomodels database in a variety of formats, accession number BIOMD0000000273 - Pokhilko2010_CircClock, and from the PlaSMo repository (www.plasmo.ed.ac.uk), accession PLM_6."

Reviewer #4

"1. Although it is clear that CRY1 and CRY2 are critical for temperature compensation at 27ºC, they appear not to contribute at 12ºC since there is no difference between rhythms of WT and cry1cry2 at this temperature. Furthermore, the period of WT appears significantly shorter under red light or red+blue light at low temperatures and rhythms under blue light at 12ºC, particularly of CAB2:LUC, approach arrhythmia. This suggests that red light signalling might be more important at low temperatures. Therefore, it seems appropriate that the authors should have investigated the effect of red light receptors (phytochromes) or ZTL, another putative blue light receptor, on temperature compensation."

We address the clear difference in clock gene rhythms at 12 and 17C in the cry double mutant in response to reviewer 1 point 15 and reviewer 2 point 6.
We have also added data describing the phytochrome mutants' temperature compensation phenotype, please see the response to the editor's comments.

"2. I am not entirely satisfied with the model validation presented in this manuscript. Testing of the predicted behavior of LHY protein and PRR9 transcript under the model does not indicate whether this is a mechanism (as suggested p 14 para 2) or a response of temperature compensation. Particular as the model is highly constrained to reproduce the experimental data. A better test of this as a mechanistic hypothesis would be to assess temperature compensation in lhy and prr9 mutants. If the model is correct, I expect that lhy and prr9 should be impaired in temperature compensation. Furthermore, the suggestion that "temperature compensation is primarily effected through temperature-dependent modulation of light input pathways into the morning loop of the clock" is misleading. The authors have not experimentally shown that there is not an involvement of the evening loop either by analysis of mutant phenotypes or extensive analysis of, for example, protein abundance of evening loop components."

Firstly, we have now added simulations and experimental data for the *cca1 lhy* double mutant which closely match experimental data (new Supplementary Figure 6). Secondly, we have included a simulation of the *prr7 prr9* mutant demonstrating that the simulation predicts a temperature

dependant period lengthening and a reduction in rhythm robustness consistent with published data and our own experimental data under BL, as we now describe in the results section below:

"Simulating the temperature dependent effects of other clock mutants
To further our tests of the temperature dependent model, simulations were carried out for known temperature compensation mutants. CCA1 and LHY have previously been identified as playing a role in temperature compensation with CCA1 functioning at low temperatures and LHY at high temperatures (Gould *et al.*, 2006). In the Pokhilko model the LHY and CCA1 are treated as a single factor called LHY (Pokhilko *et al.*, 2010). Therefore, we simulated the *lhy cca1* double mutant by reducing the translation of LHY to zero. The simulation identified that a residual short period oscillator was still functional in the double mutant and that the mutant was still capable of temperature compensation, this seemed surprising, therefore, we tested the prediction identifying a close match with experimental data, thus further validating our model (Supplementary Figure 7A-D). It also predicted the subtle reduction in rhythm robustness at lower temperatures. Similarly the *prr7 prr9* double mutant has been shown to have a temperature dependent phenotype (Salome *et al.* 2010), with period increasing and rhythm robustness decreasing with a rise in temperature. When the *prr7 prr9* double mutant was simulated by reducing the translation parameters close to zero, a temperature compensation phenotype was produced that qualitatively matched that of the published mutant (Salome *et al.,* 2010). The simulation showed period lengthening with a rise in temperature and a dampening in rhythm robustness (Supplementary Figure 7E-G). Although temperature dependent phenotype was correctly predicted, the model underestimated the absolute period value by approximately 2.5h across the temperature range This is removed in supplementary figure 7G because this underestimate follows from the dynamics of the P2010 model that models 27°C in the wild type, as this also underestimated the period of the *prr7 prr9* mutant."

"3. The authors must place this work within the context of previous experimental work on analysis of clock mutants on temperature compensation of the clock (Gould et al., 2006; Salome et al., 2010). For example, it seems to have been largely ignored in this manuscript that GI, a component of the evening loop, has previously been suggested to be involved in temperature compensation. This is surprising since this was the major finding in the previous manuscript by these authors on this topic (Gould et al., 2006). Furthermore, the phenotypes reported for various mutants in these publications might support the hypothesis put forward under the model in this manuscript. For example, Salome et al reported 'overcompensation' in prr7prr9 mutants in addition to the defects on temperature entrainment in these mutants.
The major concern is that the findings concerning LHY appear to be at odds with the authors own previous findings of Gould et al., 2006. in the current manuscript Page 11 "Our experimental data matched the prediction, as peak LHY levels were 2.7-fold higher at 27{degree sign}C than at 12{degree sign}C (Figure 3B and C)." This is the complete opposite to the finding of Gould et al 2006 "in which increases (from 17 to 27{degree sign}C), (caused) decrease for LHY" . In the current manuscript it is argued that LHY mRNA increases with temperature yet in their previous manuscript it is argued LHY decreases with temperature. This apparent discrepancy and the authors failure to address the discrepancy leave the reader uncertain about the reproducibility of the results and therefore I wonder if the interpretations are valid."

Over the last decade it has become apparent that the clock is a complex network and it is difficult for us to think sensibly of molecules acting in isolation. For this reason we took a systems approach rather than our previous functional analysis of single genes. To align our paper with Salome et al 2010, we have included a simulation of the *prr7 prr9* mutant, qualitatively matching key features of the experimental data, as described above.

To address the reviewer's other point, we now clarify the comparison to Gould et al 2006 (please see the response to point 4 below).

Note that our present results show that *LHY* mRNA levels *do not change* with temperature and LHY protein levels increase (consistent with protein data of James et al., 2012). Within the manuscript we now discuss the result in Gould et al 2006:

"Previous results showed that *LHY* transcript levels decreased with increasing temperatures (Gould *et al.,* 2006). These data were acquired from plants of the Wassilewskija (Ws) accession, grown

under white light, using a q-RT PCR primer set that detected only a subset of *LHY* gene models, missing the gene model At1g1060.4, that was absent from earlier genome annotations. Our current data is from the Col-0 accession and assayed under BL conditions, using q-RT PCR primers carefully designed to allow the amplification of all the gene models of *LHY* currently in TAIR 10. A recent publication illustrates that there are temperature dependent changes at least within a subset of splice variation events in *LHY,* (James *et al*, 2012) with spliced transcripts decreasing with increasing temperature. This temperature-induced alteration in splice variant accumulation is the most likely explanation for the differences between the data presented here and the Gould et al., 2006 data set."

"4. Why were the temperature induced changes in CCA1/LHY, GI and TOC1 levels reported previously by the authors (Gould et al., 2006) not included in the model? In the previous manuscript the authors claimed that introducing these changes in to the Lock et al., (2005) model of the circadian clock was sufficient to recapitulate the experimental temperature compensation phenotypes. If this is the case, why is it necessarry to include also the effects of blue light signaling? Do the conclusions of the current manuscript still apply if the changes in clock transcript abundance are included in the model? Or is it the authors are no longer secure in the results described in Gould et al., 2006 because of the apparent difference in the data from the current manuscript?"

To address this point, we have added this text to the beginning of the modeling section in supplementary information:

"Simulation of an earlier, two-loop clock model showed that lower *LHY* transcription, representing 27°C and tending to shorten period, could be balanced by higher transcription of the hypothetical evening loop component *Y* (representing *GI*), to control the period in the wild type. Transcript levels in this model were otherwise unconstrained, but rhythmic amplitude was shown to be sensitive to simulated mutation of *Y* (Gould et al. 2006). The period control coefficients of *LHY/CCA1* and *GI* transcription have opposite signs in the current model (Supplementary Table 3), indicating that a similar balance could, in principle, be established by these parameters in the absence of other constraints. However, *GI* and other transcript levels would change substantially with temperature, in contradiction to our data. "

"5. p 8 line 3. This sentence is misleading. There appears to be no difference between cry mutants and WT at 12ºC."

We have adjusted the text to read:
"The *cry1* mutation singularly had no detectable effect on period at 12° or 17°C under BL relative to WT"

"6. p 8 paragraph 2. The final sentence is misleading. CRY proteins could be switching between active/inactive forms in a temperature-dependent manner. While is it true that this is downstream of CRY protein accumulation, I feel the implication of this paragraph by the authors is to suggest that temperature is not having an effect on CRY activity directly, which is not necessarily true."

What we stated is "suggesting that temperature signals converge with light inputs downstream of CRY protein accumulation." This makes it very clear that a simple explanation of temperature regulating protein abundance is incorrect, and we did not make any assumptions about the activity of the protein.

"7. p 9 line 6. If LHY and CCA1 transcript levels at or below WT trough levels is the criteria for arrhythmia, then these transcripts are arrhythmic at all temperature in cry1cry2. This sentence should be revised."

We state "Clock gene expression was arrhythmic in the *cry1 cry2* double mutant at 27°C, as expected, with *LHY* and *CCA1* mRNA levels at or below the WT trough level." The sentence states two facts: one, that *CCA1* and *LHY* mRNA are arrhythmic and two that the levels of mRNA are below the WT trough. We are not making an argument that expression below trough levels is a characteristic of or criterion for arrhythmia. For an illustration, please see response to reviewer 1 point 15.

"8. Discussion, second paragraph and fifth paragraph. It is not clear to me that either a network-balancing model or a temperature-(in)sensitive component model is particularly supported over the other. It is very difficult to distinguish the two. The nature of the circadian network is such that an effect on a single component will have broad effects on the network. Furthermore, the possibility that the temperature effects are not mediated through modulation of CRY activity, for example, has not been excluded."

See earlier discussion in response to reviewer #1, points 19 and 20.

"9. Were the fluence rates of light for each light quality the same? These should be given in the methods."

This is now done, please see response to reviewer #1 point 4.

"10. Figure 1. The data-points for cry1 in most of the panels in Fig 1B are not visible. Is it possible for this to be improved for clarity?"

We have adjusted the figures reducing the symbol size to resolve this issue.

"11. Figure 2.
a. What does an mRNA level of "1" mean? It clearly cannot mean transcript levels equal to UBQ10, since this reference transcript is expressed at higher levels than CCA1, LHY and TOC1 (as previously reported by these authors and others). If the data has been normalized to a data-point, this should be described in the legend and/or methods. Also, the y-axis should be labeled as 'Relative mRNA levels". However, it is this reviewer's preference that the true transcript/UBQ10 value be given.
b. The model seems to poorly predict amplitude and shape of the oscillation in cry1cry2 12ºC and 17ºC, particularly of LHY and CCA1. Is this an artifact of the normalization of the transcript data, or does this actually provide insight into potential inadequacies in the model?"

These points are addressed in response to reviewer 1 point 15 and reviewer 2 point 8.

2nd Editorial Decision 01 August 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the two referees who agreed to evaluate this resubmitted manuscript. As you will see from the reports below, the referees felt that the revisions and new evidence presented here at had improved this work to some degree. They both have, however, substantial remaining concerns, which preclude its publication in its present form.

As you will see below, both reviewers appreciated the new experimental data presented in this work, but both reviewers raised concerns regarding the ability of the mathematical model to predict mRNA levels -- indeed the second reviewer was concerned that while the model correctly predicts low levels of LHY protein in cry1cry2 mutants, it appears to achieve this shift in protein levels via a different mechanism than the one observed in vivo.

We asked the reviewers to consider and comment on each other's points, and during this process the reviewers agreed that this was an important point and both reviewers additionally and independently suggested that the role of TOC1 in the model should be re-evaluated given recently published evidence. I include below some excerpts from their comments:

Reviewer #1 on Reviewer #2

"I do agree with the other referee in that the model still performs poorly in predicting mRNA levels for clock genes. Maybe this is based on the fact that the model includes TOC1 as an activator and not the repressor that it seems to be, or maybe it is because the parameters the authors used for their model in blue light were taken from their previous model at a different temperature.

I was also thinking back about the explanation of the significant rhythmicity in cry1cry2 double mutants in blue light at 27ʃC. I find it odd that the period is close to 24 hours, when the expected period length under these conditions should be longer (if one follows the linear regression of period length at 12 and 17ʃC). This was not addressed, and it probably should be."

Reviewer #2 on Reviewer #1

"I think that we both agree that the model does not do a good job in predicting the expression changes. I think the experimental observation of the cry1cry2 dependent phenotype is robust and interesting but that that the modeling part does not add much to the understanding of the phenomena particularly because mechanisms that seem to explain the changes in LHY protein levels in the mutant in the model vs. in planta seem to be different (translation versus transcription) Mathematical models, in general, are not able to predict all the phenomena and in my opinion these discrepancies should be pointed out by the authors and the results of the modeling toned down in the text.

My additional concern is that the new model of the Arabidopsis circadian clock published by some of the authors of this manuscript (Pokhilko et al., 2012) includes TOC1 acting as a repressor of CCA1 (a result that has been confirmed experimentally by some of the authors too, Huang et al., 2012). This manuscript uses an older version of the model as a base (Pokhilko et al., 2010) in which TOC1 acts as an activator or CCA1. Although by the time the authors had submitted the first version of the manuscript this data had not been published it does diminish the relevance of the modeling work at this point."

In general, journal policy requires that works resubmitted after rejection are clearly supported by the peer reviewers. In this case, however, while these issues clearly remain important, the editor feels that they may be addressable with a combination additional analysis and clearer discussions of the caveats of the model. As such, we would like to offer you the exceptional opportunity to prepare another final revision of this work. Both reviewers felt that changes in the function of TOC1 should be considered, and at minimum it should be determined whether updating the underlying model would substantially alter the main conclusions of this work, or improve some of the discrepancies between the model predictions and the experimental observations.

In addition, when preparing your revised manuscript, please note that we provide a new functionality that allows readers to directly download the 'source data' associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). This sort of figure-associated data may be particularly appropriate for this work, and we generally require that authors provide any numeric data that is directly compared to, or used to fit, mathematical models. Please see our Instructions of Authors for more details on preparation and formatting of figure source data (<http://www.nature.com/msb/authors/index.html#a3.4.3>).

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. We reserve the right to send any revised manuscript back to one or both of the reviewers for comment and, as you probably understand, we can give you no guarantee at this stage that the eventual outcome will be favorable.

Reviewer #1 (Remarks to the Author):

Many of my points have been addressed appropriately. It was nice to see the western blot for LHY

protein in the cry1cry2 double mutant. The discussion of point #12 (why some cry1cry2 seedlings remained rhythmic in BL) is interesting and adds to the manuscript.

For point 5: I do think it is important to include data from 22C conditions. For no other reason, it is a nice way to show that the authors' data is similar to what other labs have collected in the past. In the flowering time field, conditions often vary from one lab to the next and it is sometimes impossible to reproduce some results. The effects of vernalization come to mind for example, as some labs grow their seedlings on soil, others on plates, under various light fluences and so on.

For point 11, both sets of traces almost look the same to me; there is a bit more noise for the called arrhythmic set compared to the called rhythmic set. I wonder if starting the analysis from ZT12 might not cause FFT-NLLS to latch on to the first peak in CCR2:LUC (in both sets the first peak in LL has a strong amplitude, so one would expect both sets to be rhythmic if the above statement is true; however, I have found that FFT-NLLS is rather temperamental when calling rhythmicity). Re-running the analysis from ZT24 on would probably return mostly arrhythmic traces for cry1cry2 in BL, and would strengthen the authors' case even more. WT should not be affected.

For point 14: I disagree that the period of all components of a coupled system is the same. CAB2 and CCR2 are output genes and may be under the converging control of many signaling cascades, in addition to the clock. Period differences between reporters have been used as an argument (including by some of the co-authors of this manuscript) to suggest that CAB2, PHYB and CAT3 may be under the control of distinct oscillators.

For point 15: I still do not think that the model behaves all that well in predicting expression level changes. The normalized expression data shown in response to reviewers also raises another point: there appears to be a large phase change in cry1cry2 compared to Col-0 for CCA1 and LHY expression, but this does not translate into a phase change for CAB2:LUC or CCR2:LUC.

For point 25: when seedlings were transferred to the new conditions. This was always done at ZT0, so seedlings going to 12C had a step down while going from darkness to light. In other words, seedlings receive two contradictory cues: lights on (morning), and temperature step down (evening). Good thing nothing interesting happens at 12C.

The phyA-201 allele is in Ler, not Col-0. Maybe you mean phyA-211, I believe the reference is the same, although I do not understand why.

Page 6, middle paragraph, last sentence: there is a word missing between "eight genotype" and "2 reporter combinations".

With cry1 and cry2, the single mutants have weak phenotypes, but the cry1 cry2 double mutant is essentially arrhythmic at 27C. Similarly for phyA and phyB, the single mutants have subtle phenotypes. What about the phyAphyB double mutant?

Page 9: the discussion about the discrepancies between this and previous (Gould 2006) articles makes use of the recent James et al 2012 article. However, only Figure 1 of the James et al paper uses 12C, the rest of the paper focusing on the effects of chilling plants to 4C on alternative splicing. James et al start to see alternative splicing at 12C, but this is the low end of your conditions, so I would modulate the discussion a little.

Is it a good thing to use primers that amplify from all gene models? Only some of the gene models will form a functional protein, so including "misspliced" mRNAs might be misleading. There was also that recent Plant Cell paper about alternative splicing of CCA1 at low temperatures, where a truncated CCA1 protein negatively regulated the full length, functional protein.

Page 13: the cca1lhy double mutant has a short period, and short period mutants do not show defects in temperature compensation (in any systems I believe). I exclude gigantea mutants from the list of short period mutants, since only weak alleles show a short period, and null alleles have almost no period phenotype (although they do have a low amplitude). This is beyond the scope here, but does the model include a limit-cycle for the oscillator, with a critical threshold above which temperature compensation defects can manifest? There is some mention of limit cycles on page 18.

Also: a cca1lhy double mutant was not rhythmic in the Portoles and Mas article 2010 (PLoS Genetics). Different alleles though.

Page 15: I would remove the mention of the James et al 2012 article since it only looks at seedlings grown at 22C or shifted from 22C to 12C or 4C. Unless what the authors mean here is that alternative splicing would be evident at higher temperatures, were it not for NMD?

Page 16: another mention of the James et al 2012 article that might not be warranted. The authors do see an increase in LHY protein between 4C and 12C, but these conditions do not overlap with the conditions shown in this manuscript. Consistent: yes; confirmatory: no.

Pages 13 and 17: on page 13 the authors state that decreasing LHY translation in prr7prr9 leads to a longer period with compensation defects. prr7prr9 had higher LHY levels at higher temperatures relative to wild-type in the Salome et al 2010 article, so shouldn't this indicate an increase in LHY translation instead? Page 17 does mention that increasing LHY translation in the model lengthens period.
That higher temperatures increase LHY levels but result in a shorter period might reflect the existence of an as yet unidentified component.

Page 18: typo in second paragraph. crr1 cry2

Supplementary material:
Page 13: in the main text, the authors state that the modeling of prr7prr9 required a lower LHY translation value to reproduce the temperature compensation defect, although not to the extent seen by Salome et al 2010. But here the paragraph states that all parameters (other than PRR7 and PRR9) kept their WT values.
Are these 2 different levels in prr7prr9 modeling, or are these one and the same? If they are, then the main text is a bit misleading, because I was not expecting such a small change in period (2 hours from 12 to 27C, with a shorter period in between at 17C?).

Figure S7: too many data points in the graphs! I would replot the graphs (panels C and F) with 20% of the dots.

From answers to other reviewers:
Reviewer 2, Figure 2: were relative mRNA levels obtained from a standard curve during the qPCR, or from the delta C(t) method?

Reviewer #2 (Remarks to the Author):

The description of the modeling and experimental set up has been greatly improved in this revised version of the manuscript. The presentation of the temperature compensation data of the phytochrome mutants strengthens the specific effects on temperature compensation of the cryptochromes.

Although the mathematical model is able to predict some changes of the circadian clock at different temperatures the authors should also point out to the limitations of the model predictions. For example, the model correctly predicts the increase in LHY protein levels but unchanged LHY RNA levels at higher temperatures. It also appears to correctly predict low levels of LHY protein in the cry1cry2 mutant. However, the model, in general, does a bad job in predicting the overall RNA levels of clock components in this mutant. Therefore, the low levels of LHY proteins in cry1cry2 seedlings are likely to be caused by the extremely low LHY RNA levels. In contrast, in the model they appear to be caused by a change in the translation rate. These and other discrepancies in the model predictions should be discussed.

Minor points:
Supplemental Tables 3 and 4 do not appear complete in the PDF file.
The number of significant digits in Supplemental Tables 3 and 4 differ between columns.

1st Revision - authors' response 19 October 2012

Thank you for this further chance to modify the manuscript. Our new manuscript includes a detailed section analysing whether the altered role of TOC1 in the new Pokhilko *et al.* 2012 model would affect the matter raised by the referees. We have also gone through and made specific changes as suggested by the reviewers. We have tracked changes in the manuscript so that changes can be followed.

EDITOR (restating REVIEWER comments):

"Both reviewers felt that changes in the function of TOC1 should be considered, and at minimum it should be determined whether updating the underlying model would substantially alter the main conclusions of this work, or improve some of the discrepancies between the model predictions and the experimental observations."

In consideration of these comments we have performed a detailed analysis of the issues raised concerning the Pokhilko *et al.* 2012 clock model. We specifically analyse whether the change in function of *TOC1* could result in an improved fit of mRNA in the c*ry1/cry2* double mutant. This analysis has been added to the supplementary information in the paper and we have included a sentence in the discussion section of the main manuscript. In summary: the detailed analysis demonstrates that in this regard the new model behaves in a similar fashion to the older one and that a significantly better fit of mRNA in the *cry1 cry2* double mutant cannot be obtained.

Addressed specific points from reviewer 1.

Reviewer 1: "For point 5: I do think it is important to include data from 22°C conditions. For no other reason, it is a nice way to show that the authors' data is similar to what other labs have collected in the past. In the flowering time field, conditions often vary from one lab to the next and it is sometimes impossible to reproduce some results. The effects of vernalization come to mind for example, as some labs grow their seedlings on soil, others on plates, under various light fluences and so on."

Response: We agree in hindsight it would have been useful to generate a 22°C data set, however, the data set we generated was performed sequentially and in rapid succession, using identical seed stocks, media stocks and lighting conditions. This allows direct comparison across the whole data set. Generating a completely matched data set now for 22°C may not be possible. We would have to use new seed stocks or aged seed stocks. This may make the data difficult to directly compare. While generating the 22°C data would be a useful benchmark it does not affect the conclusions that we make in this paper.

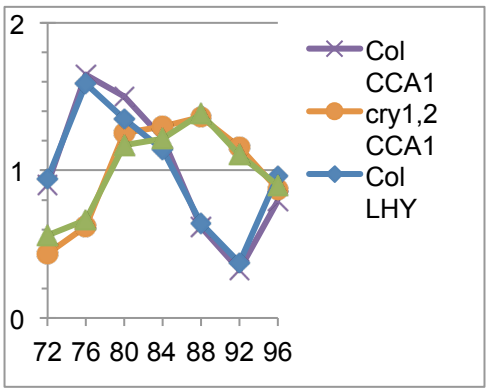
Reviewer 1: "For point 11, both sets of traces almost look the same to me; there is a bit more noise for the called arrhythmic set compared to the called rhythmic set. I wonder if starting the analysis from ZT12 might not cause FFT-NLLS to latch on to the first peak in CCR2:LUC (in both sets the first peak in LL has a strong amplitude, so one would expect both sets to be rhythmic if the above statement is true; however, I have found that FFT-NLLS is rather temperamental when calling rhythmicity). Re-running the analysis from ZT24 on would probably return mostly arrhythmic traces for cry1cry2 in BL, and would strengthen the authors' case even more. WT should not be affected."

Response: We have repeated the analysis as suggested and added a line in the text. Re-running the analysis from ZT24 does not change our conclusion. There was a clear difference between wild-type and mutant in rhythm robustness/arrhythmicity occurring with either time window

Reviewer 1: "For point 14: I disagree that the period of all components of a coupled system is the same. CAB2 and CCR2 are output genes and may be under the converging control of many signaling cascades, in addition to the clock. Period differences between reporters have been used as an argument (including by some of the co-authors of this manuscript) to suggest that CAB2, PHYB and CAT3 may be under the control of distinct oscillators."

Response: The referee is correct in saying that the period of all components of a coupled system does not have to be identical. The question here is whether the conclusions based on the two luciferase reporters and qRT-PCR are reliable. Since the effects considered are consistent across all three we feel this is clearly the case,

Reviewer 1: "The normalized expression data shown in response to reviewers also raises another point: there appears to be a large phase change in cry1cry2 compared to Col-0 for CCA1 and LHY expression, but this does not translate into a phase change for CAB2:LUC or CCR2:LUC."
Response: One cannot deduce that there is a phase change as this is in constant light. The period has changed in the double mutant and also we cannot assume that the WT and mutant clocks were in phase when the clocks were transferred from LD to LL (i.e. at ZT0).



Reviewer 1: "The phyA-201 allele is in Ler, not Col-0. Maybe you mean phyA-211, I believe the reference is the same, although I do not understand why."
Response: Corrected-see track changes

Reviewer 1: "Page 6, middle paragraph, last sentence: there is a word missing between "eight genotype" and "2 reporter combinations"."
Response: Corrected-see track changes

Reviewer 1: "With cry1 and cry2, the single mutants have weak phenotypes, but the cry1 cry2 double mutant is essentially arrhythmic at 27C. Similarly for phyA and phyB, the single mutants have subtle phenotypes. What about the phyAphyB double mutant?"
Response: This would be interesting to know but we do not have a comparable data set for the *phyA phyB* double.

Reviewer 1: "Page 9: the discussion about the discrepancies between this and previous (Gould 2006) articles makes use of the recent James et al 2012 article. However, only Figure 1 of the James et al paper uses 12C, the rest of the paper focusing on the effects of chilling plants to 4C on alternative splicing. James et al start to see alternative splicing at 12C, but this is the low end of your conditions, so I would modulate the discussion a little."
Response: Added a caveat that the James et al. paper looks at splicing over a 20°C to 4°C temperature range. See track changes

Reviewer 1: "Is it a good thing to use primers that amplify from all gene models? Only some of the gene models will form a functional protein, so including "misspliced" mRNAs might be misleading. There was also that recent Plant Cell paper about alternative splicing of CCA1 at low temperatures, where a truncated CCA1 protein negatively regulated the full length, functional protein."
Response: We agree that this is a limitation but without a full understanding of the diversity of splice variation at this point it is difficult to make a judgment as to which splice variant to follow. In our opinion what is important is that we flag the splice variation as an issue, which we have now done. This has to be taken into consideration when the community has a fuller understanding about the implications.

Reviewer 1: "Page 13: the cca1lhy double mutant has a short period, and short period mutants do not show defects in temperature compensation (in any systems I believe). I exclude gigantea mutants from the list of short period mutants, since only weak alleles show a short period, and null alleles have almost no period phenotype (although they do have a low amplitude). This is beyond the scope here, but does the model include a limit-cycle for the oscillator, with a critical threshold above which temperature compensation defects can manifest? There is some mention of limit cycles on page 18.

Response: The reviewer is incorrect to suggest that only long period mutants have temperature compensation phenotypes. We have previously published that the *lhy* and *cca1* single mutant both have temperature compensation phenotype and both have short period phenotype (Gould et al 2006).

Reviewer 1: Also: a cca1lhy double mutant was not rhythmic in the Portoles and Mas article 2010 (PLoS Genetics). Different alleles though."

The *cca1 lhy* mutant has been published as rhythmic in multiple manuscripts Alabadi *et al.* 2002, Locke et al. 2005. In the Portoles and Mas article, they use a *TOC1:LUC* marker and it is possible that TOC1 expression becomes uncoupled from the clock in the *cca1 lhy* double mutant.

Reviewer 1: "Page 15: I would remove the mention of the James et al 2012 article since it only looks at seedlings grown at 22C or shifted from 22C to 12C or 4C. Unless what the authors mean here is that alternative splicing would be evident at higher temperatures, were it not for NMD?"
Response: Here, we use James et al. as an example of a possible balancing mechanism no more.

Reviewer 1: "Page 16: another mention of the James et al 2012 article that might not be warranted. The authors do see an increase in LHY protein between 4C and 12C, but these conditions do not overlap with the conditions shown in this manuscript. Consistent: yes; confirmatory: no."

Response: We have altered the manuscript to consistent rather than confirmation.
Reviewer 1: "Pages 13 and 17: on page 13 the authors state that decreasing LHY translation in prr7prr9 leads to a longer period with compensation defects. prr7prr9 had higher LHY levels at higher temperatures relative to wild-type in the Salome et al 2010 article, so shouldn't this indicate an increase in LHY translation instead? Page 17 does mention that increasing LHY translation in the model lengthens period.

Reviewer 1: That higher temperatures increase LHY levels but result in a shorter period might reflect the existence of an as yet unidentified component."
And later:
Reviewer 1: "Page 13: in the main text, the authors state that the modeling of prr7prr9 required a lower LHY translation value to reproduce the temperature compensation defect, although not to the extent seen by Salome et al 2010. But here the paragraph states that all parameters (other than PRR7 and PRR9) kept their WT values.
Reviewer 1: Are these 2 different levels in prr7prr9 modeling, or are these one and the same? If they are, then the main text is a bit misleading, because I was not expecting such a small change in period (2 hours from 12 to 27C, with a shorter period in between at 17C?)."
Response: There appears to have been some confusion here. The *prr7prr9* mutant is modeled by setting PRR7 and PRR9 translations to zero. This is mathematically equivalent to removing PRR7 and PRR9 from the model. LHY translation is not altered from its WT value; indeed all other parameters retain their WT values. This has been made clearer in the text on page 14 where we have changed the sentence "When the *prr7 prr9* double mutant was simulated by reducing the translation parameters close to zero,.." to "When the *prr7 prr9* double mutant was simulated by reducing the translation parameters (of PRR7 and PRR9, only) close to zero,.."

Reviewer 1: Page 18: typo in second paragraph. crr1 cry2
Response: Corrected- see track changes.

Supplementary material:
Reviewer 1: Figure S7: too many data points in the graphs! I would re-plot the graphs (panels C and F) with 20% of the dots.
Response: Instead of removing data points we have reduced the sizing of the data points. This has made the graphs much easier to visualize.

Reviewer 1: From answers to other reviewers: Reviewer 2, Figure 2: were relative mRNA levels obtained from a standard curve during the qPCR, or from the delta C(t) method?
Response: Adjusted text to state "were relative mRNA levels obtained from a standard curve"
Addressed specific points from reviewer 2.

Reviewer 2: The description of the modeling and experimental set up has been greatly improved in this revised version of the manuscript. The presentation of the temperature compensation data of the phytochrome mutants strengthens the specific effects on temperature compensation of the cryptochromes.

*Reviewer 2:*Although the mathematical model is able to predict some changes of the circadian clock at different temperatures the authors should also point out to the limitations of the model

predictions. For example, the model correctly predicts the increase in LHY protein levels but unchanged LHY RNA levels at higher temperatures.

Reviewer 2: "It also appears to correctly predict low levels of LHY protein in the cry1cry2 mutant. However, the model, in general, does a bad job in predicting the overall RNA levels of clock components in this mutant. Therefore, the low levels of LHY proteins in cry1cry2 seedlings are likely to be caused by the extremely low LHY RNA levels. In contrast, in the model they appear to be caused by a change in the translation rate."

Response: The referee is questioning the value of our prediction by suggesting that we only get low LHY protein levels because we have lowered the translation rate. This is not the case as lowering the translation rate produces a system-level response that largely counters the effect of this. We can test how much effect translation has on the levels of LHY total protein by increasing the level of LHY translation from the mutant value (p1=0.19) to the original WT value (p1=0.4) while not changing any of the other cry1cry2 mutant parameters. We find that the levels of LHY total proteins do increase as p1 is increased, but they are still significantly lower than the corresponding WT levels and the mean level never gets above half way between the mutant and WT mean levels. An increase in translation parameter (p1) has a negative feedback effect on the LHY mRNA levels (via increasing the PRRs), and hence there is a trade-off in translation of a higher translation constant (p1) and lower LHY mRNA levels.

Hence, the relative difference in LHY protein levels in the mutant vs.WT is not caused by a change in the translation rate (p1). Rather, it is a result of a more complex feedback. Though high levels of mRNA (with translation parameter unchanged) will lead to higher protein levels, this is an overly simplistic view of LHY dynamics, since LHY protein has a negative feedback on mRNA levels.

Reviewer 2: Minor points:
Supplemental Tables 3 and 4 do not appear complete in the PDF file.
The number of significant digits in Supplemental Tables 3 and 4 differ between columns. ??
Response: Corrected- see track changes.

3rd Editorial Decision 04 December 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now finally heard back from referee #2. As you will see, this reviewer is globally satisfied with the modification made but still raises some important points that should be addressed. We would thus kindly ask you to revise your manuscript according to the five points below:

1. Since Pokhilko et al. 2012 is the most recent model, it would be appropriate to mention it and cited it in the Introduction.
2. The comparison between the Pokhilko 2010 and Pokhilko 2012 models should be made transparently in the Results section. In addition, we would suggest adding a table that summarizes what conditions (eg as currently listed as i)-v) on p 14 of SI) can be matched or not matched by the respective models.
3. The limitations of both models should be acknowledged in the Discussion.
4. The discrepancy between LHY mRNA levels in the cry1cry2 mutant in the model and the experiments should also be discussed.
5. The large time-series luciferase dataset produced in this study is a very valuable component of this study. We appreciate that the data is hosted at BioDare. However, for the purpose of long term archival and ease of access, we would ask you to provide the whole dataset in supplementary information as "dataset" (as text files, Excel tables, R objects, database files) in a form that allows others to re-analyzed the data, reproduce your analysis and build upon it..

Thank you for submitting this paper to Molecular Systems Biology.

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Referee reports:

Reviewer #2 (Remarks to the Author):

The authors analyzed the Pokhilko et al., 2012 model to test whether it would change their conclusions obtained with the 2010-temperature compensated model. The authors state that the 2012 model behaves " in a similar fashion to the older one".

The authors perform an analysis of the parameters of the 2012 model to find changes that could mimic five characteristics of the cry1cry2 mutant experimental data (listed on p 14, supplemental material):

i. LHY mRNA and protein levels
ii. PRR9 mRNA levels
iii. PRR7 mRNA levels
iv. TOC1 mRNA levels
v. GI mRNA levels.

The "old" 2010 - temperature compensated model gives a good approximation for characteristics ii, iv and v at 27C but not for i. Characteristic (i) states that " mutant LHY mRNA and total protein levels have to decrease and match the level of the respective WT troughs", however, the LHY mRNA levels are always significantly elevated in the model with respect to the in vivo experimental data. I did not find experimental data for PRR7 levels, point iii, so it is not known if the 2010-temperature compensated model works well for this.

Based on their conclusions I think the 2012 model does not work so well since after their analysis the authors conclude that:

" any parameter changes will have a similar effect on PRR9 mRNA and GI mRNA, hence making it impossible to match conditions (ii) and (v) simultaneously".

" From the analysis it also appears that no direct combination of the parameters could lead to the match of the mutant data in TOC1 mRNA" .

In addition it is unclear if the low LHY mRNA and protein levels can be achieved simultaneously with the other four characteristics. The authors only comment that it cannot be achieved with the required low PRR7 mRNA (point iii).

Reading their analysis I cannot agree that new model behaves similarly to the old one. In any case this analysis should be presented in the results section, currently it is only briefly mentioned in the discussion. The description of the analysis is confusingly written and could be improved. The limitations of the modeling strategy should be pointed out in the results section.

I had mentioned that in my opinion it seemed that the changes in LHY protein levels in the 2010-temperature compensated model in the cry1cry2 mutant could be caused by a change in LHY translation rate. I thank the authors for the subsequent analysis showing that this is not necessarily the case. This explanation would be useful for the readers.

However, I believe the authors should still discuss the apparent discrepancy between LHY mRNA levels in the cry1cry2 mutant in the model and the experiments. The fact that in both cases LHY protein levels are increased might indicate that both systems (model and in vivo) might be working differently.

2nd Revision - authors' response 09 January 2013

Thank you for this further chance to modify the manuscript. We have as you suggested revised our manuscript based on the 5 points you highlighted:

1. Since Pokhilko et al. 2012 is the most recent model, it would be appropriate to mention it and cited it in the Introduction.

We have now added:
"This model has been recently modified to add the evening complex (EC), comprised of LUX (LUX ARRHYTHMO), ELF3 and ELF4 (Pokhilko *et al.,* 2012). The binding of the EC to the promoters of the target genes PRR9 and LUX suppresses their transcription (Nusinow *et al*, 2011). In addition, TOC1 has been changed from an activator to a repressor (Gendron *et al.* 2012; Huang *et al.*, 2012; Pokhilko *et al.,* 2012). Both of the abovementioned mathematical models are based solely on data from plants grown in standard laboratory conditions close to 22°C. They are able to match much of the experimental data including altered circadian phenotypes of mutated components,"

2. The comparison between the Pokhilko 2010 and Pokhilko 2012 models should be made transparently in the Results section. In addition, we would suggest adding a table that summarizes what conditions (eg as currently listed as i)-v) on p 14 of SI) can be matched or not matched by the respective models.

This has been done on page 13 in the results section where the following has been added:

"The model also predicted that in the *cry1 cry2* double mutant LHY protein levels at 27°C would be low and arrhythmic. This raises the question of whether the low protein levels could be an outcome of low *LHY* mRNA levels. Interestingly, the mathematical model suggests that this is not the case since model *LHY* mRNA levels at 27°C, though lower than peak WT levels, are still not nearly as low as the data suggests, Figure 2.

We have analysed whether switching to the more recent clock model of (Pokhilko et al, 2012) would lead to improvement of the fits of the mathematical model to data. In particular, it is natural to ask whether the experimentally confirmed negative feedback of *TOC1* on *LHY* mRNA would aid this. We conclude that switching models would lead to some improvement of the fits (e.g. that of *LHY* mRNA), but at the expense of others. The resulting trade-offs are presented in Table 2. Most importantly, an attempt to create a WT temperature compensated clock model from (Pokhilko et al, 2012) would potentially not be able to capture one of the two striking features of the WT data at lower temperature: the increasing period at the lower temperature and the decreasing PRR9 mRNA. A detailed description of the analysis to back up these statements (and those in Table 2) is given in Supplementary Information."

The following table summarising the comparison has also been added.

Characteristic	Fit of temperature-dependent model at 27°C	Improvements by switch to (Pokhilko et al. 2012) model
LHY mRNA and protein	mRNA levels too high compared to data while protein levels show a good match.	Can be improved at the expense of worse fit for two out of three following mRNAs: PRR9, TOC1 and GI. Choice of which two depends on choice of parameters.
PRR9 & TOC1 mRNA	good	
GI mRNA	shape is good but levels are too low	Any improvement comes at the expense of the PRR9 mRNA fit.

Table 2

3. The limitations of both models should be acknowledged in the Discussion.

We have added the following to the discussion:
"Inevitably both mathematical models suffer from limitations that arise from lack of knowledge and uncertainty about specific connections in the system (Pokhilko 2012 SI). In particular, the identity and detailed mechanism of the assumed acute light activation of LHY, PRR9 and GI transcription after dawn remains to be clarified. In addition, the modelling of the sequential activation of the

transcription of the PRRs by each other and by LHY/CCA1 protein to reproduce the experimentally-observed wave of PRR9, PRR7, PRR5 (NI) inhibitors is only partially supported by data (Farre et al, 2005) and remains to be elucidated. The mechanistic details of the inhibition of LHY/CCA1 by TOC1 are currently unknown. Recent data suggest the involvement of additional proteins in the regulation of gene expression by LHY and CCA1 (Lau et al, 2011) and these are not included in these models. Although they are included in the 2012 model, the exact mechanisms of the formation of the protein complexes between ELF3 and ELF4, ELF3 and GI, and between ELF3, ELF4, LUX (EC) and their functional significance need to be further elucidated. In the 2012 model it is assumed that post-translational regulation of ELF3 protein complexes is by different forms of the COP1 ubiquitin E3ligase. The details of this are unknown and the dynamics of COP1-containing complexes awaits further elucidation. Improvement of future mathematical models will rely on improved understanding of interactions as above and on measurements of the stoichiometry, modification, dynamics and abundance of multi-protein complexes."

4. The discrepancy between LHY mRNA levels in the cry1cry2 mutant in the model and the experiments should also be discussed.

This has been put together with the analysis discussed in the response to 2 above as an introduction for the comparison between P2010 and P2012 models (page 13).

5. The large time-series luciferase dataset produced in this study is a very valuable component of this study. We appreciate that the data is hosted at BioDare. However, for the purpose of long term archival and ease of access, we would ask you to provide the whole dataset in supplementary information as "dataset" (as text files, Excel tables, R objects, database files) in a form that allows others to re-analyzed the data, reproduce your analysis and build upon it.

BioDare supports data storage, sharing, public distribution, visualisation and, critically, rhythmic data analysis by multiple algorithms. Access is provided by a biologist-friendly web browser interface and programmatically via web services. BioDare currently has >70 users, with >70,000 numerical time- series and >10,500,000 data points. While no database can be completely future proof it is part of our BBSRC grants remit that data is made publically available for at least 10 years after a the grant. Moreover, data can be readily download as CSV files that can be used directly by MATLAB, R, Python and Excel. We have added a table to the supplementary information with the BioDare experimental ID. We have also download the CSV files for each experiment and added them as data sets.

Specific responses to the comments of the second referee.

1. The second referee states that he/she cannot agree that new model behaves similarly to the old one and requests that the analysis should be presented in the results section, rather than being briefly mentioned in the discussion.

It is a little unfair of the referee to say that we claimed that the new model behaves similarly to the old one. The actual statement that we made was highly qualified and restricted the similarity to the ability of the second model to obtain a better fit than the first. However, we have taken all the referee's comments to heart and we have amended the paper along the lines requested. (i) As described above there is now a description of the analysis in the results section on page 13. (ii) The description of the analysis in the SI has been improved and clarified. It has been simplified by expressing the analysis directly in terms of the change in the solution when parameters are changed (i.e. in terms of dg/dk) rather than indirectly in terms of principal components. (iii) Supplementary figures 7-11 (bottom two panels) have been improved. (iv) The proof that P2012 can't do a better job at fitting the data than P2010 model has been rewritten and we have extended the analysis on fitting the cry1cry2 mutant at 27C and added analysis on fitting the WT at low temperatures. We amended a statement about the effect of significant changes of multiple parameters on TOC1.

2. The referee states that "it is unclear if the low LHY mRNA and protein levels can be achieved simultaneously with the other four characteristics. The authors only comment that it cannot be achieved with the required low PRR7 mRNA (point iii)."

This point is now dealt with in the last paragraph of page 15 of the SI.

3. The referee points out that he/she did not find experimental data for PRR7 levels even though we previously mentioned it as part of our analysis.

He/she is right to point this out and it was unnecessary for the analysis. We are not using any data for PRR7.

