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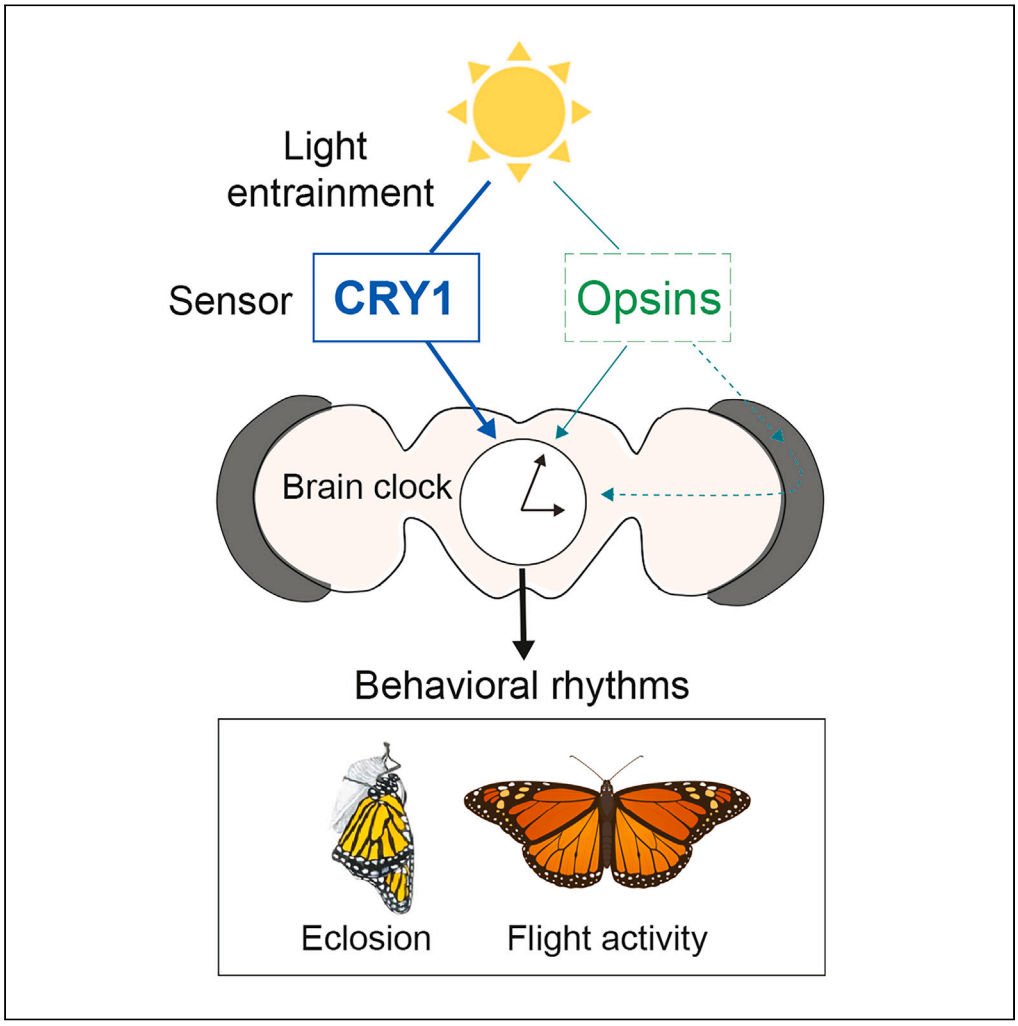

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Highlights

Monarch *dpCry1* mutants display damped eclosion and brain molecular circadian rhythms

Temperature cycles re-entrain eclosion and molecular rhythms in *dpCry1* mutants

Robustness of rhythmic adult flight activity is decreased in *dpCry1* mutants

Genetic impairment of opsin-based photoreception impacts flight suppression at night

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Loss of functional *cryptochrome 1* reduces robustness of 24-hour behavioral rhythms in monarch butterflies

Samantha E. Iiams,[1,2] Guijun Wan,[1,3,4] Jiwei Zhang,[1,4] Aldrin B. Lugena,[1] Ying Zhang,[1] Ashley N. Hayden,[1] and Christine Merlin[1,2,5,*]

SUMMARY

Light is one of the strongest cues for entrainment of circadian clocks. While some insect species rely only on visual input, others like *Drosophila melanogaster* use both the visual system and the deep-brain blue-light photoreceptor cryptochrome for entraining circadian rhythms. Here, we used the monarch butterfly *Danaus plexippus* (*dp*), which possesses a light-sensitive *cryptochrome 1* (*dpCry1*), to test the conservation of mechanisms of clock entrainment. We showed that loss of functional *dpCry1* reduced the amplitude and altered the phase of adult eclosion rhythms, and disrupted brain molecular circadian rhythms. Robust rhythms could be restored by entrainment to temperature cycles, indicating a likely functional core circadian clock in *dpCry1* mutants. We also showed that rhythmic flight activity was less robust in *dpCry1* mutants, and that visual impairment in *dpNinaB1* mutants impacted flight suppression at night. Our data suggest that dpCRY1 is a major photoreceptor for light-entrainment of the monarch circadian clock.

INTRODUCTION

Cryptochromes (CRYs) are an ancient class of blue-light and ultraviolet (UV)-sensitive flavoproteins present in a wide range of organisms[1–3] that evolved from photolyases responsible for the repair of UV-generated pyrimidine dimers.[4,5] Although CRYs have retained the N-terminal photolyase-related (PHR) domain, which contains the chromophore access cavity for binding of the flavin adenine dinucleotide (FAD) cofactor, they have lost the DNA repair capability of a photolyase[1–3] and classically function in the circadian system. Two types of CRYs, type 1 CRYs and type 2 CRYs, can be differentiated based on their circadian function. Type 2 CRYs, present in mammals and all insects studied so far with the notable exception of *Drosophila melanogaster* (*dm*) and other Schizophoran flies,[6] function as circadian repressors of the CLOCK (CLK):BMAL1 heterodimeric circadian transcription factor complex to generate circadian rhythms.[7–12] In contrast, type 1 CRYs, including *Drosophila* dmCRY, are light-sensitive and appear to function in the entrainment of the core circadian clock.[13–15] In type 1 CRYs, the photolyase homology region contains a group of tryptophan (Trp) residues essential for enabling the photosensing capabilities of CRY by reducing the FAD chromophore into its active state,[16–20] likely driving conformational changes which allows CRY to initiate downstream signaling pathways.[21–24]

Drosophila dmCRY has been firmly established as the primary blue-light circadian photoreceptor for resetting the circadian clock cell autonomously to the daily light:dark (LD) cycle. dmCRY is rapidly degraded after light exposure and mediates the degradation of one of its partners, TIMELESS (dmTIM), in a light-dependent manner.[13,25] A point mutation altering a key residue for FAD binding, named cry^{baby} (cry^{b}), leads to severe entrainment defects, including a loss of light-dependent dmTIM degradation and the inability to phase-shift circadian response to light pulses.[13,14] Curiously, cry^{b} mutants and a fly cry^{0} full knockout were shown to still retain robust behavioral rhythms in adult emergence from their pupal case and locomotor activity, and were also entrainable to new LD regimes,[14,26] suggesting the existence of other CRY-independent photoreceptors and pathways for entraining *Drosophila* circadian behaviors. This was confirmed by rendering the fly clock completely blind after eliminating all photoreceptors with a double mutant for cry^{b} and *glass*, a transcription factor necessary for the development of photoreceptors in the eyes and Hofbauer-Buchner eyelets.[27] Further characterization has shown that in addition to dmCRY, signal transduction pathways utilizing phospholipases encoded by *no receptor potential A* (*norpA*) and *phospholipase C* (*Plc21C*), as well as several rhodopsin (Rh) photoreceptors, Rh1, Rh5, Rh6, and Rh7 acting either in the eyes or the brain, play a role in *Drosophila* clock entrainment.[28–31] Whether such a complex network is also required for circadian clock entrainment in other insect species remains unknown.

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To date, the role of light-sensitive CRY in clock entrainment in other insects has been limited to transgenic expression of monarch and mosquito CRY1s in *Drosophila*[15,32] and *in vitro* studies in *Drosophila* Schneider 2 cells and in a monarch butterfly, *Danaus plexippus* (*dp*), embryo-derived DpN1 cell line that contains a light-driven clock.[11,15] Yet, the extent of CRY1's contribution to *in vivo* eclosion and activity rhythms remains unexplored in species outside *Drosophila*. Here, using a previously generated *dpCry1* loss-of-function monarch knockout,[33] we genetically demonstrated that dpCRY1 is involved in monarch daily and circadian rhythms. Loss of functional *dpCry1* significantly damped (i.e., reduced amplitude) circadian adult eclosion behavior and abolished molecular circadian rhythms in the monarch brain. We showed that robust circadian eclosion rhythms can be restored by entrainment to temperature cycles (TCs) in *dpCry1* mutants but not in the core clock component *dpClk* null mutants. Importantly, we also showed that rhythmic flight activity was less robust in *dpCry1* and in visually impaired *dpNinaB1* mutants, which despite presenting diurnal patterns of activity, displayed striking phenotypes. Together, these data suggest that light-entrainment of the monarch behavioral rhythms may rely on both dpCRY1 and additional photoreceptors.

RESULTS

dpCry1 loss-of-function monarch mutants exhibit damped circadian eclosion rhythms and disrupted molecular rhythms in the brain

To investigate whether the role of light-sensitive CRY in circadian behavioral rhythms entrainment is conserved among insect species *in vivo*, we started by testing if a *dpCry1* loss-of-function mutation, a 2-bp deletion in the fourth exon that led to no detectable production of the corresponding truncated protein that would otherwise lack the Trp-containing C-terminal domain,[33] would affect monarch circadian eclosion rhythms. Eclosion, the emergence of the fully mature adult from its chrysalis, is currently the only known behavior in monarchs that is both under circadian control and easily tractable in laboratory conditions via recording with infrared cameras.[14,34] Monarchs *dpCry1* wild-type (*dpCry1*$^{+/+}$) and homozygous knockout (*dpCry1*$^{-/-}$) siblings were placed on the day of pupation in 12 h:12 h LD at 25°C and then released into constant darkness (DD) one to two days prior to their expected eclosion. As expected, *dpCry1*$^{+/+}$ monarchs exhibited circadian eclosion rhythms with a peak in the number of monarchs emerging around the anticipated timing of lights on (CT0), as previously described[8,12,34,35] (Figures 1A and 1B). In contrast, adult eclosion rhythms of *dpCry1*$^{-/-}$ were significantly damped (Figures 1A and 1B). This result stands in sharp contrast with findings in *cry*^{0} null mutant flies, which exhibit circadian pupal eclosion as robust as that of wild-type flies.[26] Quantification by qPCR of mRNA levels of two core clock genes, *period* (*dpPer*) and *timeless* (*dpTim*), in monarch brains over the circadian day revealed that both genes cycled robustly in brains of wild-type monarchs but were expressed arrhythmically and at constitutively high levels in brains of *dpCry1*$^{-/-}$ (Figure 1C). Together, these results indicate that loss of functional dpCRY1 has a profound effect on monarch eclosion rhythms, either through a loss of circadian timekeeping or most likely through a lack of synchronization among rhythms of individual pupae due to loss of dpCRY1-mediated photoreception and synchronization.

Temperature cycles re-entrain eclosion and molecular rhythms in *dpCry1* knockouts

To determine whether eclosion behavior and brain molecular rhythms observed in *dpCry1*$^{-/-}$ mutant monarchs could be entrained by a potent zeitgeber other than light, we sought to re-entrain the mutants to TC, as in flies.[14] Monarchs were raised in 12 h:12 h LD at 25°C from eggs to the end of the fourth larval instar stage and subjected to constant light (LL) conditions for the duration of the fifth larval instar to eliminate any core clock oscillations from larval entrainment to light before entraining pupae to TCs[34] (Figure 2A). On the day of pupation, pupae were moved to a 12 h-15°C: 12 h-25°C TC in DD, in anti-phase to the LD cycle under which larvae were previously entrained, i.e., with low temperature coinciding to the previous light phase and high temperatures to the previous dark phase, until eclosion (Figure 2A). We found that both *dpCry1*$^{+/+}$ wild-type and *dpCry1*$^{-/-}$ mutant monarchs eclosed rhythmically with a strong peak at the beginning of the warm phase after the temperature rose from 15°C to 25°C (Figure 2B, *left*), suggesting that eclosion behavior could be re-entrained to TC even in the absence of a functional dpCRY1. The possibility that the observed TC entrainment was caused by an acute response to the increase in temperature, i.e., masking, cannot however be excluded. We also performed the same experiment using loss-of-function mutants of a core clock gene activator, *clock* (*dpClk*)[35] and their wild-type siblings. In contrast to wild-type monarchs, which eclosed rhythmically within the first 2 h of the warm phase, *dpClk*$^{-/-}$ failed to display high amplitude rhythms and eclosed randomly throughout the warm phase. Although masking may be occurring in *dpClk*$^{-/-}$ as it may in *dpCry1*$^{-/-}$, these data suggest that functional core clock proteins are necessary for the synchronization of monarch eclosion to TCs (Figure 2B, *right*). To test if re-entrainment of behavioral rhythms by TC would be mirrored at the molecular level, we quantified *dpPer* and *dpTim* mRNA levels over the 12 h-15°C: 12 h-25°C TC in DD in the brain of both adult wild-type and *dpCry1*$^{-/-}$ mutants. We found that *dpPer* and *dpTim* mRNA rhythms were robustly entrained by TC in the brains of wild-type monarchs, with peaks of expression at the transition from warm to cold phases and troughs in the early warm phase (Figure 2C). Importantly, a similar trend was observed in the brains of *dpCry1*$^{-/-}$ mutants, where *dpTim* exhibited rhythms indistinguishable from those in wild-type brains and *dpPer* also cycled albeit with a lower amplitude than in wild-type (Figure 2C). Although these results do not unequivocally demonstrate that the core circadian clock is functional in the *dpCry1*$^{-/-}$ mutant, which would require a formal assessment under constant conditions, they indicate that the core molecular components of the *dpCry1*$^{-/-}$ monarch clock can still function to drive transcriptional rhythms under TCs. Thus, the unexpected significant damping of eclosion rhythms under LD conditions in *dpCry1*$^{-/-}$ mutants suggests the intriguing possibility that dpCRY1 may function as a major light-entrainment pathway of circadian rhythms in monarchs at the pupal stage.

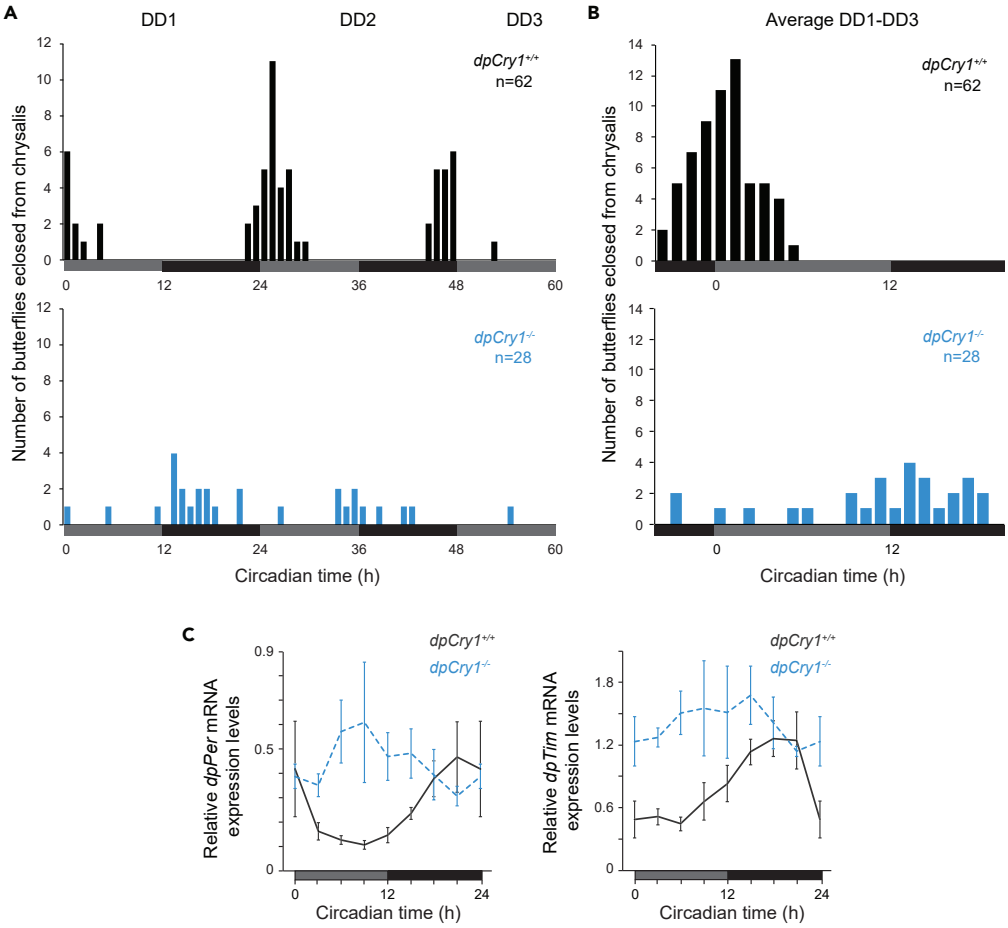

Figure 1. Monarch butterfly *dpCry1* knockouts exhibit damped circadian rhythms in eclosion behavior and disrupted brain mRNA clock genes rhythms

(A) Profiles of adult eclosion in constant darkness (DD) of wild-type ($^{+/+}$; dark gray) and *dpCry1* homozygous mutant siblings ($^{-/-}$; blue) entrained to 12 h:12 h light/dark (LD) cycles at 25°C for the first 7 days of pupation. Eclosions across DD1, DD2, and DD3 are binned in 1 h intervals. Horizontal gray bars: subjective day; black bars: subjective night.

(B) Data from A pooled together and binned in 1 h intervals. Kolmogorov-Smirnov (KS) test maximum difference between $dpCry1^{+/+}$ and $dpCry1^{-/-}$: $p < 0.0001$. COSINOR: $dpCry1^{+/+}$, $p = 0.00001$; $dpCry1^{-/-}$, $p < 0.005$.

(C) Circadian expression of *period* (*dpPer*) and *timeless* (*dpTim*) in the brain of adult monarchs eclosed from A and re-entrained for at least 7 days to 12 h:12 h LD cycles at 25°C. $DpCry1^{+/+}$: solid dark gray lines; $dpCry1^{-/-}$: dashed blue lines. Values are mean ± SEM of four brains for each timepoint. MetaCycle with period ranging from 21 h to 27 h: *dpPer* in $dpCry1^{+/+}$, adjusted (adj) $p = 0.0000019$; *dpPer* in $dpCry1^{-/-}$, adj $p = 0.086$; *dpTim* in $dpCry1^{+/+}$, adj $p = 0.00011$; *dpTim* in $dpCry1^{-/-}$, adj $p = 0.31$.

Daytime patterns of adult flight activity are affected in *dpCry1* and in blind *dpNinaB1* knockouts

Because the visual system and CRY-independent light entrainment pathways may not be fully developed until after adult emergence in monarchs, we sought to test the relative contribution of dpCRY1 and other photoreceptors on locomotor rhythms in adults. To this end, we used custom-built flight mills to assess daily patterns in flight activity of adult $dpCry1^{-/-}$ mutants and wild-type siblings. Individual monarchs were tethered and suspended to one end of a balanced mill arm placed inside a plastic barrel allowing them to freely fly on a horizontal, circular plane, in 15 h: 9 h LD conditions at 21°C. Activity was recorded over 3 days using an infrared beam placed above the butterfly's rotational flight path, and the distance flown was calculated based on the number of revolutions per 1 h time-bins. LD conditions were chosen over DD as monarch flight activity is inhibited in the absence of light (not shown), impeding formal tests of circadian activity. Without the ability to assess true circadian rhythms of flight behavior, and in absence of flight activity at night, we focused our analysis on the time-of-day-dependent changes in activity exhibited during the 15 h light phase. We observed a notable impact from loss of functional *dpCry1* on a rhythmic pattern of flight behavior that occurs within the light phase in LD conditions. In contrast to wild-type monarchs, which flew in a rhythmic pattern within the 15 h light phase with a strong peak in the middle of the day, $dpCry1^{-/-}$ exhibited damped rhythms of daytime activity with a period of ramping activity in the morning but more evenly distributed activity compared to controls over the three days of recordings in average (Figure 3A and 3B; Table S1). These results indicate that dpCRY1 also plays an important role in driving activity rhythms in adult monarchs.

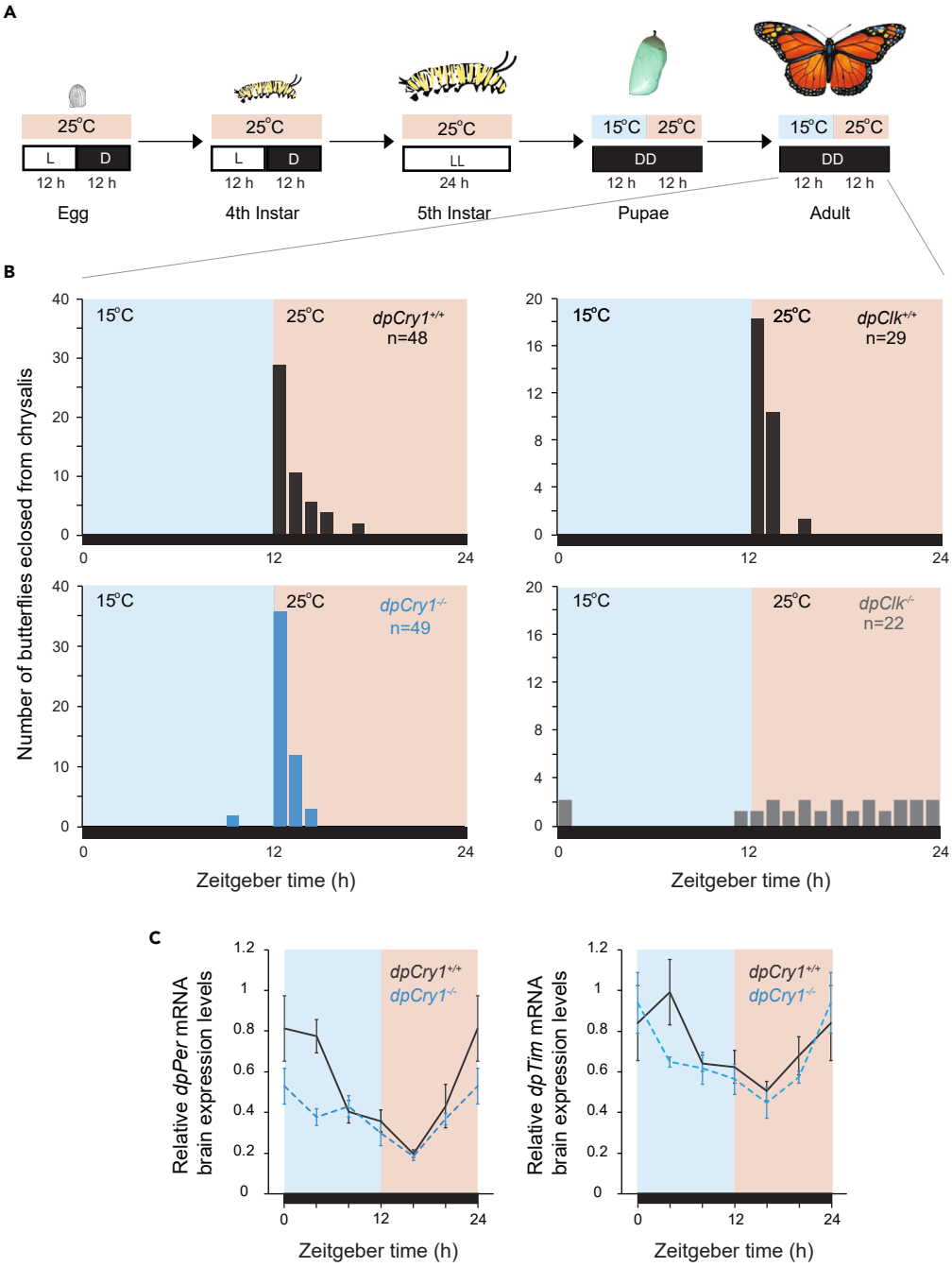

Figure 2. Temperature cycles re-entrain rhythmic eclosion behavior and brain molecular rhythms in *dpCry1* knockouts

(A) Schematic of entrainment by LD and temperature cycles at different developmental stages from egg to adult. Monarchs were raised under 12:12 LD cycles at 25°C from egg to fourth instar larvae, transferred into constant light (LL) during the fifth instar, and then placed in constant darkness (DD) in temperature cycles at 15°C for 12 h and 25°C for 12 h throughout pupal and adult stages.

(B) Profiles of adult eclosion after pupal entrainment for ~10 days to 12 h-15°C/12 h-25°C cycles. Black horizontal bars: constant darkness. (*Left*) Profiles of $dpCry1^{+/+}$ (*top*, dark gray) and $dpCry1^{-/-}$ siblings (*bottom*, blue). KS test maximum difference between genotypes: p = 0.639. One-way ANOVA between $dpCry1^{+/+}$ and $dpCry1^{-/-}$: p = 0.016. (*Right*) Profiles of $dpClk^{+/+}$ (*top*, dark gray) and $dpClk^{-/-}$ siblings (*bottom*, light gray). KS test maximum difference between genotypes: p < 0.0001. One-way ANOVA between $dpClk^{+/+}$ and $dpClk^{-/-}$: p = 0.005.

(C) Expression of *dpPer* and *dpTim* in the brain of adult wild-type and *dpCry1* knockouts monarchs eclosed from B after at least 7 days of adult entrainment to 12 h-15°C/12 h-25°C in DD. $DpCry1^{+/+}$, solid dark gray lines; $dpCry1^{-/-}$, dashed blue lines. Values are mean ± SEM of four brains for each time point. MetaCycle with period of 24 h: *dpPer* in $dpCry1^{+/+}$, adj p = 0.000087; *dpPer* in $dpCry1^{-/-}$, adj p = 0.002; *dpTim* in $dpCry1^{+/+}$, adj p = 0.0083; *dpTim* in $dpCry1^{-/-}$, adj p = 0.0059.

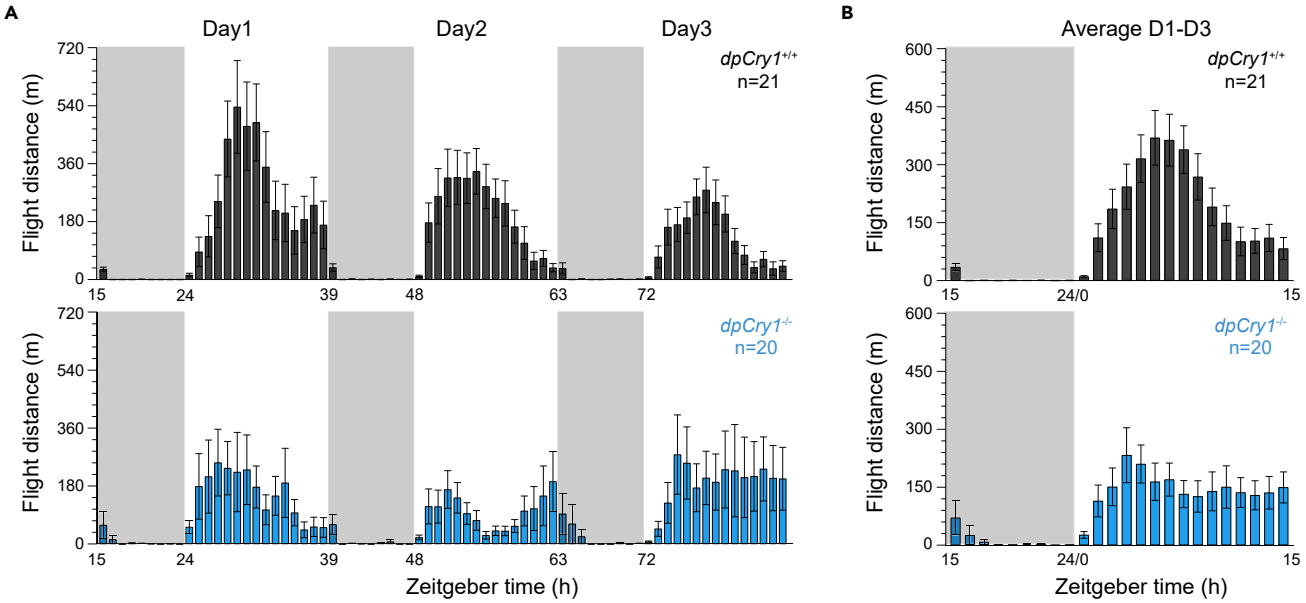

Figure 3. Rhythmic pattern of flight activity exhibited by wild-type monarchs during the light phase is damped in *dpCry1* knockouts

(A) Profiles of flight activity in 15 h:9 h LD cycles over three consecutive days measured as distance flown in a flight mill by *dpCry1*$^{+/+}$ (dark gray, *top*) and *dpCry1*$^{-/-}$ siblings (blue, *bottom*). Data are binned in 1 h intervals. Gray shading: night; no shading: day.

(B) Averaged flight activity over the three days of recording. Legends are as in (A). Meta2d rhythmicity analysis for the 15 h daytime flight period: *dpCry1*$^{+/+}$, adj $p < 0.0001$; *dpCry1*$^{-/-}$, adj $p = 1$. See also Figure S1 and Table S1.

We also tested whether other light-sensing pathways could contribute to the time-of-day-dependent changes in monarch flight activity. To determine the possible contribution of photoreceptors in the compound eyes, we blocked the light input to the eyes of wild-type and *dpCry1*$^{-/-}$ mutant monarchs by painting them with black enamel paint.[36,37] We found that, similar to that of unpainted monarchs, wild-type monarchs with black painted eyes presented a rhythmic pattern of activity with a peak in the middle of the day (Figures S1A and S1B). *DpCry1*$^{-/-}$ monarchs with black painted eyes showed damped rhythms of daytime activity that resemble those observed in unpainted *dpCry1*$^{-/-}$ (Figures S1A and S1B). These results indicate that photoreceptors in the compound eye may only play a minor role, if any, in entraining monarch flight activity rhythms, but that another photoreceptor may contribute to entrainment in *dpCry1*$^{-/-}$ mutants.

Another key light-sensing pathway in *Drosophila* circadian photoentrainment has recently been shown to rely on the deep-brain rhodopsin 7 (Rh7).[31] Although single mutants for either fly *dm*Cry or *dm*Rh7 only exhibited minor defects on photoentrainment, the double mutant was impaired with increased period length in locomotor activity and percentage of flies exhibiting arrhythmicity. To test whether a deep-brain opsin could also contribute to light-entrainment of activity rhythms in monarchs, we tested the flight activity of a *dpNinaB1* loss of function mutant. In this mutant, the production of retinal, the chromophore that renders opsins light-sensitive, is impaired in both eyes and brain resulting in individuals that are blind, including to seasonally changing photoperiods.[36] We found that both wild-type and *dpNinaB1*$^{-/-}$ mutant monarchs exhibited daily rhythms in flight activity with the expected peak during midday (Figures 4A and 4B; *top two panels*). However, we observed a notable increase in nighttime flight activity in the *dpNinaB1*$^{-/-}$ mutants. To then test if loss of function of both dpCRY1 and dpNINAB1 would have additive or synergistic effects on activity rhythms, we recorded daily flight activity of double mutants. As compared to rhythmic controls, *dpCry1*$^{-/-}$;*dpNinaB1*$^{-/-}$ exhibited damped time-of-day dependent flight activity changes during the light phase and also a lack of repression of flight at the onset of darkness, suggesting that daytime flight and nighttime flight may be differentially regulated by separate photoreceptor pathways (Figures 4A and 4B; *bottom two panels*; Table S1). Furthermore, the increase in nighttime activity observed in both *dpNinaB1*$^{-/-}$ and *dpCry1*$^{-/-}$;*dpNinaB1*$^{-/-}$ mutants compared to controls raises the intriguing possibility that the vitamin A pathway and/or an opsin is involved in repressing activity at night or in promoting sleep (Figures 4A and 4B).

DISCUSSION

Over the past 15 years, comparative analyses of clock genes in insects have revealed a greater diversity of circadian clock molecular make-ups within insects than that of the pioneering insect model *Drosophila melanogaster*.[10,11,15,38] A distinctive feature that has attracted much attention was the presence of a mammalian-like type 2 CRY in non-Drosophilid insect species, including lepidopterans and mosquitos, that functions as a circadian transcriptional repressor.[8,10,12] Less attention has been paid to the diversity of light-entrainment pathways. While type 1 CRY has been lost in some insect species, including the honeybee *Apis mellifera* and the flour beetle *Tribolium castaneum*, it is still found in others like moths, butterflies, and mosquitos.[10,39,40] Whether type 1 CRYs function as circadian photoreceptors in concert with other

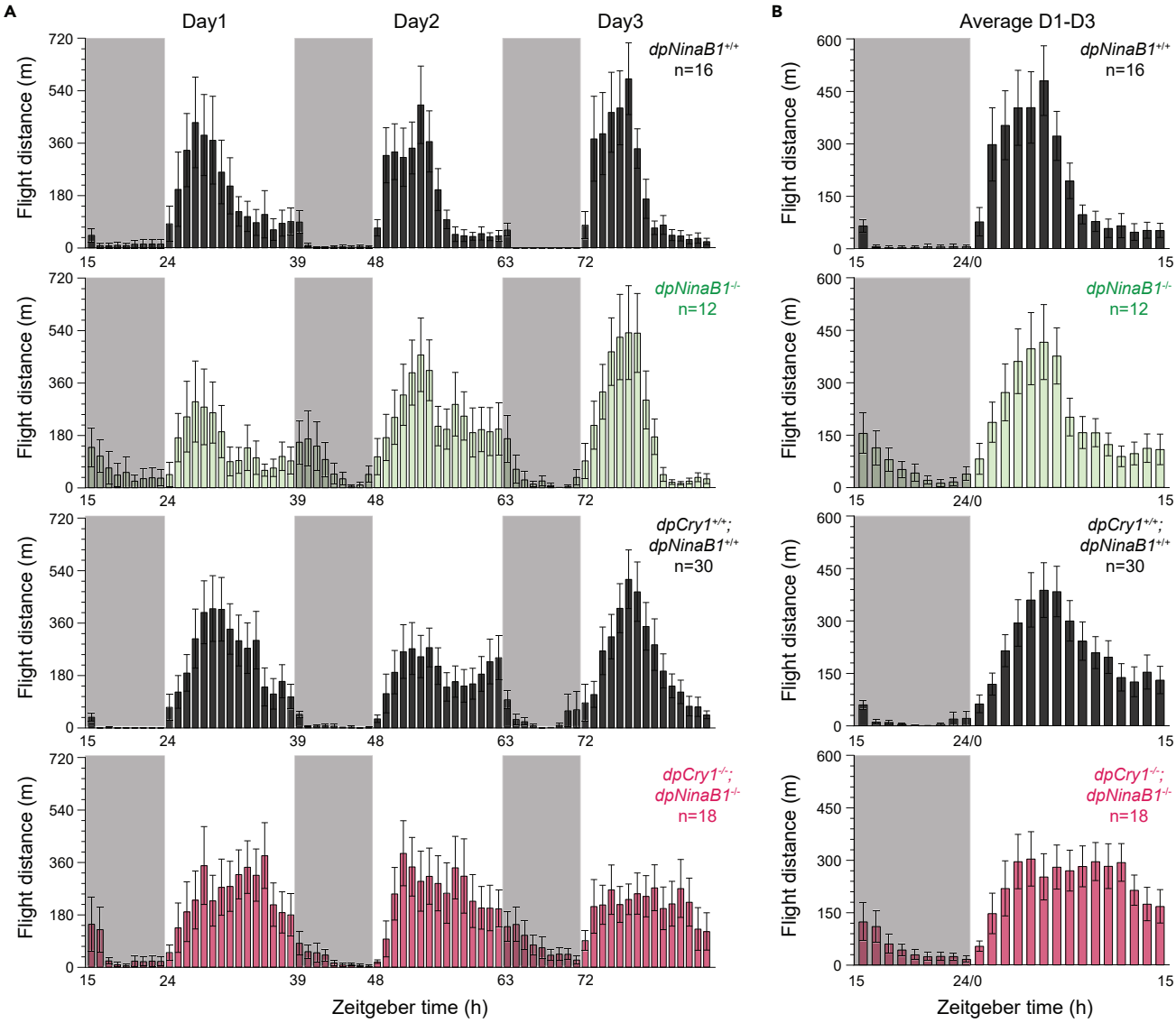

Figure 4. Genetic loss of opsin-based photoreception affects daily flight activity of monarchs

(A) Profiles of flight activity in 15 h:9 h LD cycles over three consecutive days measured as distance flown in a flight mill by blind *dpNinaB1*$^{-/-}$(green), *dpCry1*$^{-/-}$;*dpNinaB1*$^{-/-}$ double homozygous knockouts (pink), and their corresponding wild-type siblings (*dpNinaB1*$^{+/+}$ and *dpCry1*$^{+/+}$;*dpNinaB1*$^{+/+}$ respectively, black). Data are binned in 1 h intervals. Gray shading: night; no shading: day.

(B) Averaged flight activity over the three days of recording. Legends are as in (A). Meta2d rhythmicity analysis for the 15 h daytime flight period: *dpNinaB1*$^{+/+}$, adj $p < 0.0001$; *dpNinaB1*$^{-/-}$, adj $p < 0.0001$; *dpCry1*$^{+/+}$; *dpNinaB1*$^{+/+}$, adj $p < 0.0001$; *dpCry1*$^{-/-}$; *dpNinaB1*$^{-/-}$, adj $p = 0.002$. See also Table S1.

signaling pathways for the light-entrainment of daily and circadian behaviors in non-Drosophilid species remains unknown, as our current knowledge has remained limited to *Drosophila melanogaster*.[13,14,26–31]

To our surprise, characterizing the relative importance of the ortholog of dmCRY in the entrainment of 24 h rhythms in the monarch butterfly revealed a fundamental difference in the relative contribution of CRY for the *in vivo* light-entrainment of the circadian clock in insects. Unlike *Drosophila cry* null mutants, in which behavioral rhythmicity persists even at the pupal stage due to additional dmCRY-independent rhodopsin photoreceptors and signal transduction pathways for light-entrainment of the circadian clock,[26,28,29,31] monarch *dpCry1* knockouts exhibited significantly damped pupal eclosion rhythms even after stable entrainment to LD conditions. While this apparent lack of robust rhythmicity could indicate a function for dpCRY1 in the core clock mechanism, at least at this developmental stage, it could also reflect a lack of synchronization among individually rhythmic pupae due to lack of dpCRY1-mediated photoreception, as eclosion assays measure rhythms at the populational level. The rescue of robust rhythmic eclosion and molecular rhythms of *dpPer* and *dpTim* in adult brains by TC entrainment in this mutant suggest that *dpCry1* knockouts are deficient for light-entrainment. Although

eclosion behavior was tightly linked to the increase in temperature in the *dpCry1* mutant entrained to TC, molecular rhythms of *dpPer* and *dpTim* exhibited anticipatory phases of decreasing expression starting in the cold and continuing well into the warm phase before increasing again. In the absence of free-running conditions, we cannot exclude the possibility that the circadian pacemaker requires dpCRY1 or that the effects seen with TC-entrainment result from masking. However, in *Drosophila*, CRY functions as a light sensor and is not required for pacemaker function in most tissues, and molecular rhythms in whole heads are restored in *Cry* mutant flies by TC, and this even under constant conditions after temperature entrainment.[14,41] More likely than not, the same is true in monarchs, particularly since monarchs have a dedicated pacemaker cryptochrome (dpCRY2).[8,15] Future experiments assaying monarch eclosion rhythms under different T-cycles, i.e., LD cycles delivered across a range of non-24 h periods,[42] could help validate a role of dpCRY1 in entrainment as they would be expected to produce changes in the phase angle of entrainment. The ability to entrain circadian clocks to TCs has been documented both in insects[14,43,44] and mammals,[45,46] and has been found to be mediated by nocte and the Ionotropic Receptor 25a (IR25a) in *Drosophila*.[47,48] Orthologues of these temperature signal mediators are present in the monarch genome (data not shown), and their role in temperature entrainment could also be assessed in future experiments via the generation of CRISPR/Cas9-mediated loss-of-function mutants.

The significant damping of circadian rhythms in eclosion in *dpCry1* mutants support the idea that dpCRY1 functions as a major photoreceptor for entrainment of the pupal monarch clock to light *in vivo*. In contrast, *Drosophila cry* null mutants not only maintained robust rhythms in adult activity, but also eclosed with a ~24 h rhythm, suggesting that CRY-independent pathways are functional by the time of adult eclosion, at least in this species.[26] Developmental studies of the fly visual system have revealed that the optic nerve connects to the circadian clock early in the embryonic stage and that *Rh5*, *Rh6*, and *norpA* are already expressed within larval retina cells.[49] It is still unknown whether the severe damping of monarch rhythmic eclosion behavior in *dpCry1* mutants results from dpCRY1-independent, opsin-based, light sensing pathways not yet functional in the monarch pupal brain, but this remains a formal possibility. To assess the effect of *dpCry1* mutants on 24 h rhythms at the adult stage, when all light-sensing pathways are presumably developed, we established a new behavioral assay to assess flight activity. As previously observed anecdotally, wild-type monarchs remain almost completely inactive at night. However, flight activity during the light phase exhibited time-dependent changes with a peak near mid-day in wild-type monarchs. Consistent with the damping observed in eclosion rhythms, the peak of flight activity during the light phase was also damped in the *dpCry1* mutants.

Surprisingly, blocking light input to the compound eyes with black paint did not appear to affect wild-type rhythms, suggesting that CRY-independent light sensing pathways in the compound eye, such as those utilized in *Drosophila*, may not be playing a significant role in the regulation of daily rhythmic flight activity in monarchs. Since monarchs do not fly in darkness, their flight activity with eyes painted black may seem paradoxical. However, unlike monarchs in the dark, monarchs with eyes painted black are otherwise illuminated during the light phase. It is conceivable that light perceived through extra-retinal photoreceptors expressed outside the visual system, such as those expressed in the deep brain,[13,31] could promote flight activity. Our data also indicate that a loss-of-function mutation in *dpNinaB1*, the gene encoding the rate-limiting enzyme in the vitamin A pathway responsible for the conversion of β-carotene into retinal, does not impact daytime patterns of flight activity. Because these mutants are blind and lack a functional vitamin A pathway in the brain,[36] this indicates that the contribution of brain retinal-opsins in the regulation of time-of-day dependent changes in flight activity during the light phase must be minimal, thus highlighting dpCRY1 as a major contributing photoreceptor for driving high amplitude changes in flight activity during the photophase. However, in contrast to wild-type monarchs that become inactive almost immediately upon lights off, *dpNinaB1* mutants continued to exhibit flight activity well into the night. This unexpected finding may suggest that a retinal-opsin plays a role in regulating the repression of monarch flight activity in darkness. One possible mechanistic explanation for this mutant phenotype is that the lack of retinal could leave the relevant opsin in a constitutively active state, as observed in vertebrates,[50,51] prolonging flight activity into the night. Identifying the opsin(s) involved in repressing night-time monarch flight activity, among the ones present in the monarch genome, will be necessary to eventually test this hypothesis. Together, our data suggest the existence of at least two separate photoreceptor signaling pathways functioning in the monarch brain to drive rhythmic flight activity; one that relies on dpCRY1 to drive high amplitude time-of-day dependent changes in adult flight activity during the photophase, and another that likely employs a retinal-opsin to sense the onset of darkness and repress flight activity at night.

The combined damping of eclosion rhythms, impacts on time-of-day dependent changes of adult flight activity during the photophase, and disruption of molecular rhythms in *dpCry1* mutants suggests that entrainment of monarch activity rhythms *in vivo* may rely more strongly on dpCRY1 photoreception than is the case in *Drosophila* which relies as strongly on both dmCRY and other rhodopsins/phospholipase-based signal transduction pathways. By uncovering significant species-specificity in light-entrainment mechanisms between insect species, our work underscores a need for future mechanistic studies in a greater number of insect/invertebrate species to better understand the evolution of entrainment of circadian rhythms by light across invertebrates.

Limitations of the study

Adult monarch butterflies do not exhibit significant locomotor or flight activity in constant darkness, even after being entrained to light:dark regimens for at least seven days, an amount sufficient to entrain circadian rhythms in brain clocks. The lack of sustained flight activity rhythms in constant dark conditions thus precluded analyses of truly circadian properties of flight activity. Future investigations leveraging our discovery that flight activity increases during the dark phase in the absence of a functional vitamin A pathway may prove useful in identifying conditions in which such studies will be possible. The use of dim light during the night and bright light during the day could prove useful to

investigate prolonged free-run in flight activity under constant dim light. Finally, the use of T-cycles and shifted LD cycles in future experiments would allow to comprehensively assess light entrainment of monarch flight rhythms.

STAR★METHODS

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

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.108980.

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AUTHOR CONTRIBUTIONS

S.E.I. and C.M. designed the research; S.E.I., G.W., J.Z., A.B.L., Y.Z., A.N.H., and C.M. performed research; S.E.I., G.W., J.Z., A.B.L., and C.M. analyzed data; and S.E.I. and C.M. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
0.5X RNA later	Invitrogen	AM7020
SuperScript II Reverse Transcriptase	Thermo Scientific	18064
iTaq Universal SYBR Green Supermix	Bio-Rad	1725120
Critical commercial assays		
RNeasy Mini kit	Qiagen	74104
Experimental models: Organisms/strains		
Danaus plexippus Cry1$^{-/-}$ homozygous mutants	Merlin Lab	N/A
Danaus plexippus Cry1$^{+/+}$ wild-type siblings	Merlin Lab	N/A
Danaus plexippus Clk$^{-/-}$ homozygous mutants	Merlin Lab	N/A
Danaus plexippus Clk$^{+/+}$ wild-type siblings	Merlin Lab	N/A
Danaus plexippus NinaB1 $^{-/-}$ homozygous mutants	Merlin Lab	N/A
Danaus plexippus NinaB1$^{+/+}$ wild-type siblings	Merlin Lab	N/A
Danaus plexippus Cry1$^{-/-}$*;NinaB1*$^{-/-}$ double homozygous mutants	Merlin Lab	N/A
Danaus plexippus Cry1$^{+/+}$*;NinaB1*$^{+/+}$ wild-type siblings	Merlin Lab	N/A
Oligonucleotides		
Forward primer for *period;* 5′-AGTGAAGCGTCCCTCAAAACA-3′	Markert et al.,[35] 2016	N/A
Reverse primer for *period*; 5′-TGGCGACGAGCATCTGTGT-3′	Markert et al.,[35] 2016	N/A
Forward primer for *timeless;* 5′-GGACGGCAGCGGATACG-3′	Markert et al.,[35] 2016	N/A
Reverse primer for *timeless*; 5′-CGCCGTTTCGCACACA-3′	Markert et al.,[35] 2016	N/A
Forward primer for *Rp49;* 5′-TGCGCAGGCGTTTTAAGG-3′	Markert et al.,[35] 2016	N/A
Reverse primer for *Rp49*; 5′-TTGTTTGATCCGTAACCAATGC-3′	Markert et al.,[35] 2016	N/A
Software and algorithms		
One-way ANOVA and Tukey's HSD Calculator	https://www.icalcu.com/	https://www.icalcu.com/stat/anova-tukey-hsd-calculator.html
Two-way ANOVA - Free Statistics Software (Calculator)	Holliday, 2019[52]	https://www.wessa.net/rwasp_Two%20Factor%20ANOVA.wasp
Kolmogorov-Smirnov test	Kirkman, 1996[53]	http://www.physics.csbsju.edu/stats/KS-test.n.plot_form.html
MetaCycle	Wu et al., 2016[54]	https://cran.r-project.org/web/packages/MetaCycle/index.html
Other		
Enamel based clear paint	Model master clear top coat; Testors	no. 2736
Enamel based black paint	Glossy black; Testors	no. 1147
Night vision security cameras	Night Owl Security	L-45-4511

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Christine Merlin at cmerlin@bio.tamu.edu.

Materials availability

This study did not generate new unique reagents.

Data and code availability

(1) Data reported in this paper will be shared by the lead contact upon request.
(2) This paper does not report original code.
(3) Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mutant monarch butterfly strains

Monarch *dpCry1* knockouts used throughout the experiments, *dpClk* knockouts used in the temperature cycle entrainment paradigm, and *dpNinaB1* knockouts used for flight activity assays were generated in prior studies via CRISPR/Cas9-mediated targeted mutagenesis.[33,35,36] *dpCry1;dpNinaB1* double knockouts and wild-type siblings were generated by crossing the *dpCry1* and *dpNinaB1* mutant strains to obtain and intercross double heterozygotes. Both sexes were used in all experiments.

MONARCH BUTTERFLY HUSBANDRY

For all experiments listed, monarch larvae were reared on a semi-artificial diet and adults were manually fed a 25% honey solution every day, as previously described.[35]

For testing circadian eclosion behavior, wild-type and dp*Cry1* homozygous mutant monarchs were raised at 25°C with 70% humidity under the following light:dark cycle (LD) conditions: 15 h: 9 h LD from eggs to fifth instar larvae, 12 h: 12 h LD from days 1–7 of pupation, followed by two to three days of constant dark (DD). Pupae were transferred to DD after 7 days of pupation because while some eclosed as expected on the 9th DD2, others eclosed on the first or third day of DD. For quantification of gene expression in the adult brains, adults eclosed from this experiment were re-entrained to 12 h:12 h LD cycles for at least 7 days before brains were dissected under red light in the first day of DD, as previously described.[36]

For testing eclosion behavior under TC in DD, wild-type, *dpCry1* and *dpClk* homozygous mutant monarchs were raised from eggs to fourth instar larvae in 12 h:12 h LD at 25°C with 70% humidity, transferred to constant light conditions (LL) at same temperature and humidity levels for the duration of the fifth instar, and then moved at pupation to DD in a 12 h-15°C/12 h-25°C temperature cycle in anti-phase to the previous larval LD cycle (i.e., 12 h of exposure to 25°C occurs in the larvae's previous dark phase and 12 h of exposure to 15°C occurs in the larvae's previous light phase). For quantification of gene expression in the adult brains, adults eclosed from this experiment were maintained in the same 12 h-15°C/12 h-25°C cycles in DD for at least 7 days before brain dissection.

For recordings of daily flight activity, monarchs were raised under 15 h: 9 h LD cycles at 25°C from eggs to adult emergence before being transferred in a room maintained under the same lighting conditions but at 21°C. A room was used in absence of a large enough temperature-controlled walk-in that could accommodate flight mills. In addition, the choice of a 15 h photophase, which is the natural photoperiod monarch butterflies are exposed to in summer, was motivated by the fact that it would provide a greater temporal resolution for observing flight activity rhythms given that monarchs do not fly at night. Adults were entrained in these conditions for at least 7 days before flight activity recordings.

METHOD DETAILS

Real-time qPCR

For the CT course experiment, the brains of adult wild-type and *dpCry1* homozygous mutants were entrained to at least 7 days of 12 h: 12 h LD cycles and dissected during the first day of DD at CT CT0, CT3, CT6, CT9, CT12, CT15, CT18, and CT21 under red light in 0.5X RNA later (Invitrogen).

For the temperature entrainment time course experiment, brains of adult wild-type and *dpCry1* homozygous mutants were entrained to at least 7 days of 12 h–15°C/12 h-25°C in DD before being dissected at zeitgeber time ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20.

For both experiments, RNA extractions and gene expression quantifications were performed as previously described,[36] using validated monarch *per*, *tim*, and control *rp49* primers.[35] The data were normalized to *rp49* as an internal control and normalized to the mean of one sample within a set for statistics.

Eclosion behavior assays

Adult eclosions were recorded inside an incubator using a video-tracking system, as previously described.[8,35] For circadian eclosion assays, pupae were raised for 7 days in 12 h: 12 h LD cycles at 25°C and released in DD 1 to 3 days prior to emergence, as defined by the appearance of melanization on legs visible through the pupal case. Eclosion was observed on the first, second day, and third day of DD. For temperature cycle-entrained eclosion assays, eclosion was observed between 10 and 15 days after pupation while being continuously entrained to 12 h-15°C: 12 h-25°C in DD. The developmental delay in these conditions was caused by the overall temperature decrease. Eclosion data were analyzed and plotted as 1 h bins.[35,36]

Adult flight activity assays

Rhythmic monarch flight activity was measured using custom-built flight mills individually placed inside plastic barrels, in which individual monarchs were suspended by a tether glued to their thorax on one end of the flight arm counterbalanced by a weight on the other side and allowed to freely fly in a circular horizontal plane, as previously described.[33] The number of revolutions of the arm at a time resolution of 1 min was automatically recorded using an infrared beam connected to an automatic recording counter system. Each genotype of monarchs was tested blindly under a 15 h: 9 h LD cycle at 21°C. All monarchs were acclimated for at least 3 h before the three days of motor activity tests and were fed daily with 25% honey solution ~15 min at ZT12. Data were collected using a commercial data acquisition software (CHMBDD, MB-96) and the distance flown by each individual was calculated based on the number of revolutions per hour and the radius of the circular flight path, as previously described.[33]

QUANTIFICATION AND STATISTICAL ANALYSIS

Eclosion behavior and brain molecular rhythms

p-values were calculated using one-way ANOVAs followed by post-hoc analyses, Kolmogorov-Smirnov tests, and COSINOR tests using online calculators at https://www.icalcu.com/stat/anova-tukey-hsd-calculator.html, https://cosinor.online/app/cosinor.php, https://www.wessa.net/rwasp_Two%20Factor%20ANOVA.wasp,[52] and http://www.physics.csbsju.edu/stats/KS-test.n.plot_form.html.[53]

Adult flight activity rhythmicity

For each genotype, the flight data taken for 3 consecutive days for each individual butterfly tested were considered as technical replicates, while all butterflies tested were treated as biological replicates. Flight activity in monarchs becomes suppressed in darkness and as such true circadian rhythms cannot be assessed. In LD, the pattern of activity in the light phase (from ZT0 to ZT15 in our experiments) itself exhibits time-of-day dependent changes with a peak in the middle of the day. Analysis of changes of pattern of flight activity during the photophase was performed using the Meta2d function of MetaCycle, which incorporates ARSER, JTK_CYCLE and Lomb-Scargle to detect rhythmic signals from two-dimensional time-series datasets[54] (Table S1). A meta2d analysis with default parameters except for the "minper" and "maxper", which were both fixed at 15, was performed on the average of technical replicates for each butterfly. Due to uneven number of biological replicates between genotypes, rhythmicity tests were performed separately for each genotype.