Supporting Online Material

Materials and Methods

Animals.

Monarch butterflies were housed in the laboratory in glassine envelopes in Percival incubators with controlled temperature (21°C), humidity (70%), and lighting. The butterflies were fed 25% honey every third day. Migratory butterflies were captured by Fred Gagnon near Greenfield Massachusetts (latitude 42º59'N, Longitude 72º60'W) and Carol Culler near Eagle Pass, Texas (latitude 28º71'N, longitude 100º49'W).

Flight behavior.

For flight orientation behavior, migrant butterflies were tethered and flight behavior was monitored using a modified Mouritsen and Frost flight simulator (S*1*), as described previously (S*2, S3*). Butterflies were flown outdoors under sunny skies in Worcester, MA (latitude 42º16'N, longitude 71º49'W), when the sun could be seen from their position in the flight barrel between 1100 to 1500 hours EST from 24 September through 16 November 2008. Only butterflies flying continuously for a period of at least 5 min and presenting a directional flight path (Z-scores > 500; (S*4*)) were considered for all analyses. Butterflies were taken out of the simulator after 10 minutes and not re-used. For each group, data were analyzed to determine the significance of orientation and the mean direction using the Rayleigh test, and comparisons between groups were done using the Watson-Williams test for two samples (S*5*).

For free-flight behavior, butterflies showing oriented flight were tested after their tethers were removed. Experiments were carried out indoors, in a room illuminated by artificial white ceiling lights. Butterflies were kept in a small flight cage for 30 min prior to the experiment. Each butterfly was taken out of the cage and then released at a height of about 1.75 m from the floor; each butterfly was flown three times for 20 sec each. Butterfly flight behaviors were divided into three subjective categories: (i) Normal upward flight in which butterflies initiate flight immediately after release and present a sustained flight upward from the release point; (ii) sustained horizontal flight in which when released, butterflies do not fly upward, but instead maintain sustained flight at the same height of the release point; and (iii) crashes in which butterflies fail to fly and crash to the floor. Except in the few cases of crashes, none of the

butterflies tested presented apparent defects in flight control or maneuvers. The type of behavior each butterfly presented was scored based on observations made over three trials each.

Proboscis extension reflex.

In monarchs, the proboscis extension reflex is a response in which stimulation of the chemoreceptors on the middle legs with sugar solution (unconditioned stimulus, US) results in full extension of the proboscis (unconditioned response). Individual butterflies were harnessed in 15ml polypropylene conical tubes with their head, antennae and middle legs extended through a hole at the end of the tube. Proboscis extension tests were conducted by touching the middle leg with a cotton-tipped applicator soaked in 50% sucrose solution (wt/wt). Only butterflies that showed a consistent proboscis extension reflex were used in experiments. Individuals were kept harnessed and fed daily ad libitum with 50% sucrose solution. Prior to stimulus conditioning, individuals were starved for 24 hours and checked for a positive proboscis extension reflex. Butterflies were conditioned to neutral olfactory and color stimuli (conditioned stimulus, CS) by presenting the stimulus for 5 sec (CS only), contacting the middle legs with sucrose solution for 10 sec (CS +US), and removing the stimulus after 5 sec (US only). Individuals were then held for 10 min and this pairing procedure was performed again. US-CS pairing continued in this way until the individual extended its proboscis upon the initial presentation of the CS. At this time, individuals were held for 10 min and then tested by consecutively presenting an alternate stimulus (either a different odor or color) to the butterfly for 10 sec each followed by the CS. Butterflies were deemed to have a positive PER (conditioned response) if they fully extended their proboscis in response to the CS but not the alternate stimulus. Lavender was used as the olfactory conditioned stimulus and geranium was used as the alternate stimulus. Yellow was used as the color conditioned stimulus and blue was used as the alternate stimulus. Stimuli were presented at the end of a 15cm wooden stick approximated 2cm from the head.

Removal of the antennae.

Antennae were removed by clipping with scissors at the base of the flagellum just above the pedicel (Fig. S1).

Painting the antennae.

Antennae were painted from the tip to the base of the flagellum (Fig. S1) with enamel-based clear paint (Model master clear top coat; Testors no. 2736) or black paint (Glossy black; Testors no. 1147). Antennae were dipped into the appropriate paint to cover the entire surface of

the flagellum and its base was painted with a paintbrush under a dissecting scope and dried with a cool airflow generated by a hairdryer. The completeness of painting was verified post-hoc under a dissecting scope.

The light-blocking property of the paints was tested by measuring the irradiance through a sheet of plastic that was painted or not painted and illuminated with white light, using an Ocean Optics USB 2000 fibre optic spectrometer.

Culture of antennae.

Antennae from monarch butterflies were entrained at least for five days in LD. They were then removed, and washed two times with PBS (Invitrogen) followed by five washes with Grace's insect medium (Invitrogen) supplemented by 10% fetal bovine serum (Invitrogen) and 1X antibiotics/antimycotics (Invitrogen) to remove and/or prevent potential contaminants. Antennae were then cultured individually in Grace's insect medium supplemented by 10% fetal bovine serum and 1X antibiotics/antimycotics. All the procedure was performed during the light phase of the LD cycle. For light-induced TIM degradation assay, the two isolated antennae from the same butterfly were entrained for three days in phase-reversed LD cycles, and the antennae were then collected at Zeitgeber time (ZT)6 and ZT18. For time course experiments, isolated antennae were entrained for two days in LD followed by constant darkness, and the antennae were collected every 3-hour during the second day of LD and during the first day in constant darkness.

Tissue collection.

For RNA and protein extractions, tissues (brains free from eye photoreceptors, photoreceptor layers of the eye, antennae, legs, thoraces and abdomens) were dissected and stored at -80°C until use. Brains and photoreceptor layers used for RNA preparation were dissected in 0.5X RNA later (Ambion) to avoid RNA degradation.

Real-time PCR.

Total RNA was extracted using RNeasy Micro kit (Qiagen) from various tissues dissected from monarch butterflies entrained to at least five LD cycles, either in LD or in constant darkness. For extractions from thorax and abdomen, an additional acidphenol-chloroform purification step was added before ethanol precipitation to remove lipids and proteins that block the column. Total RNA was treated with RQ1 Dnase (Promega), and random hexamers (Promega) were used to prime reverse transcription with Superscript II (Invitrogen), all

according to the manufacturers' instructions. The quantifications of clock gene expression were done using real-time quantitative PCR by TaqMan probes with an ABI Prism 7000 SDS (Applied biosystems). PCR reactions were assembled by combining two master mixes. The first mix contained approximately 1 μg of cDNA template and 13 μl of Platinum Quantitative PCR SuperMix-UDG w/ROX (Invitrogen) per reaction and was aliquoted into a PCR plate. The second mix contained forward and reverse primers (0.9 μM final concentration of each), probe (0.25 μM final concentration), and the water needed to bring each reaction to a final volume of 25 μl, and was subsequently aliquoted into the PCR plate. The monarch *per*, *tim*, *cry1*, *cry2* and control *rp49* primers and probes were identical to those reported previously (S*6*). The other primers and probes were as follows (F, forward primer; R, reverse primer; P, probe; all 5'-3'): monarch *LWRhF*, CGCGGCGCTGTATGC; *LWRhR*, GGACACGTTGTCGTCAGATGA; *LWRhP*, FAM-AGGTTCCCAGCTCTGTCATGCCAAGC-TAMRA; monarch *BlueRhF*, GCCATCAACCACCCCAGA; *BlueRhR*, CTTCACGGACTCCCATCCA; *BlueRhP*, FAM-AGGGCTGAACTTCAGAAACGTGTGCC-TAMRA; monarch *UVRhF*, CTGCATCGATCCTTGGGTTT; *UVRhR*, GCATGCGACGCTGAAGCT; *UVRhP*, FAM-TGCAATCAGTCATCCCAAGTACAGGCA-TAMRA. All primers and FAM-TAMRA labelled probes were purchased from Integrated DNA Technologies (Coralville). The efficiency of the amplification and detection by all primer and probe sets were previously validated by determining the slope of Ct versus dilution plot on a 3 x 10^4 dilution series. Individual reactions were used to quantify each RNA level in a given cDNA sample, and the average Ct from duplicated reactions within the same run was used for quantification. The data for each gene in a given tissue were normalized to *rp49* as an internal control and normalized to the mean of one time point (for temporal profiling experiments) within a set for statistics.

Western-blotting.

Tissues dissected from monarch butterflies or isolated antennae maintained in culture were homogenized in protein extraction buffer (150mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.5% NP40, 100mM NaF, Complete Protease Inhibitor Tablet (Roche)). Protein concentrations were normalized by Coomassie Reagent (Pierce) and either 20 μg (for dpPER and dpTIM) or 5 μg (for dpCRY1 and dpCRY2) of each protein sample was loaded per lane. Western blots were probed with the primary antibodies guinea pig anti-dpPER40 (1:3000), anti-dpTIM47 (1:3000), anti-dpCRY1.37 (1:3000), anti-dpCRY2.51 (1:3000) (S*6*) and monoclonal mouse anti-α-tubulin

(1:20000). Secondary antibodies were horseradish peroxidase-conjugated donkey anti-guinea pig IgG (Jackson Immunoresearch Laboratories; 1:10000) and horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz; 1:16000). Films and chemiluminescent blots were imaged with the FUJIFILM LAS-1000, and bands were quantified using the ImageGauge V4.22 software. The level of each clock protein was normalized by the level of α-tubulin and expressed relative to one sample of the set.

Supporting Figures

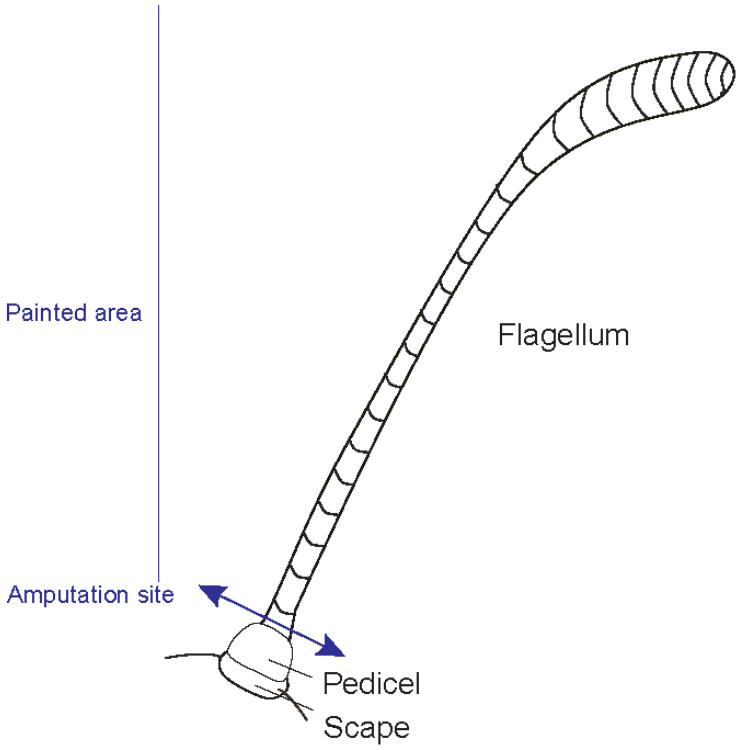

Figure S1. Anatomy of monarch butterfly antenna.

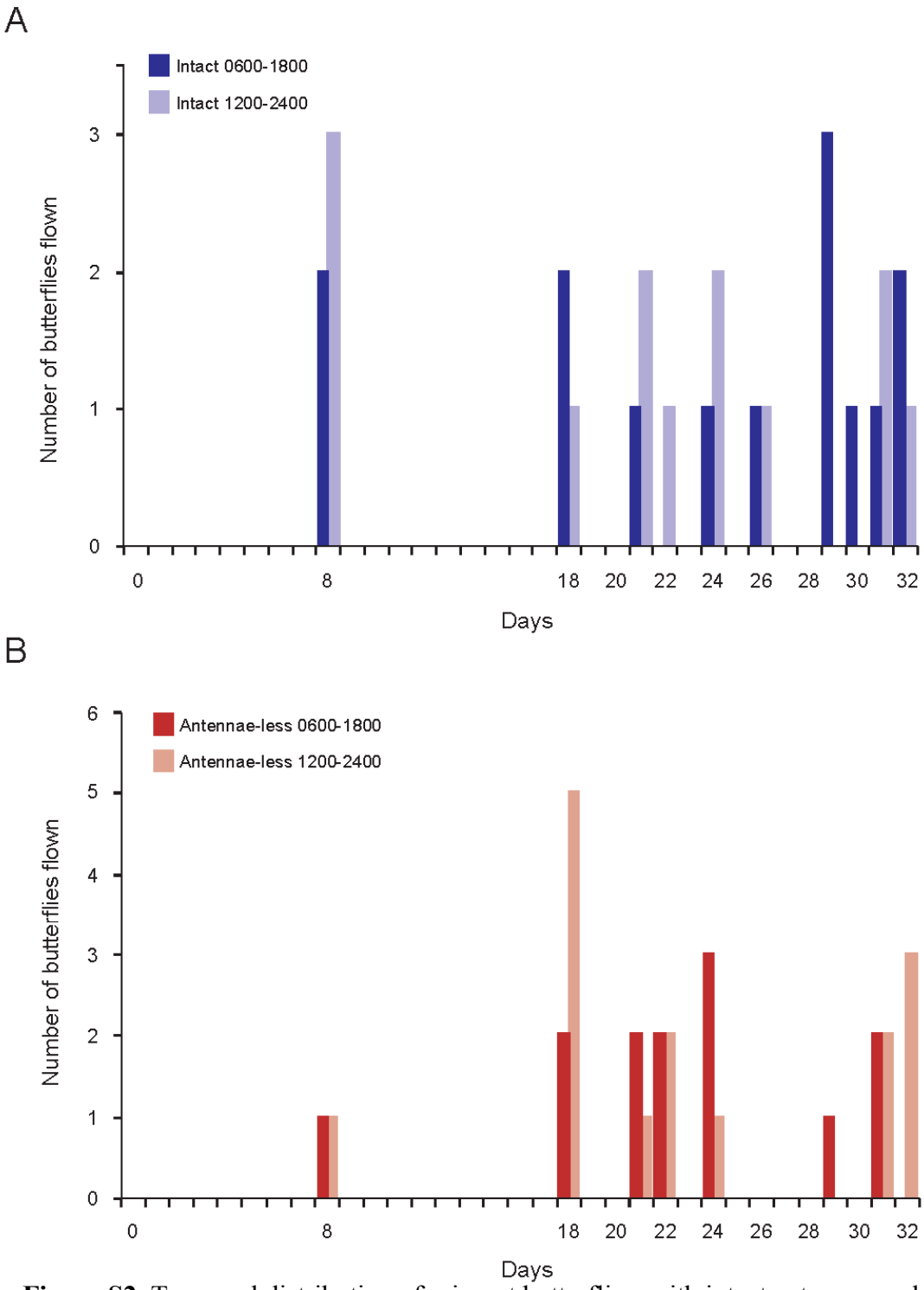

Figure S2. Temporal distribution of migrant butterflies, with intact antennae and without antennae, flown over the course of the study. Day 0 corresponds to the time of antennae removal and the beginning of entrainment to two different LD conditions (lights on from 0600 to 1800 hours and lights on from 1200 to 2400 hours) for both intact and antennae-less butterflies. (A) Controls with intact antennae entrained in the two different LD cycles. (B) Antennae-less migrants entrained in the two different LD cycles. The gap between day 0 and day 7 corresponds to the time necessary for entrainment in each lighting condition, and the gap between day 8 and day 18 corresponds to a period of inclement weather not permissive to flight simulator experiments.

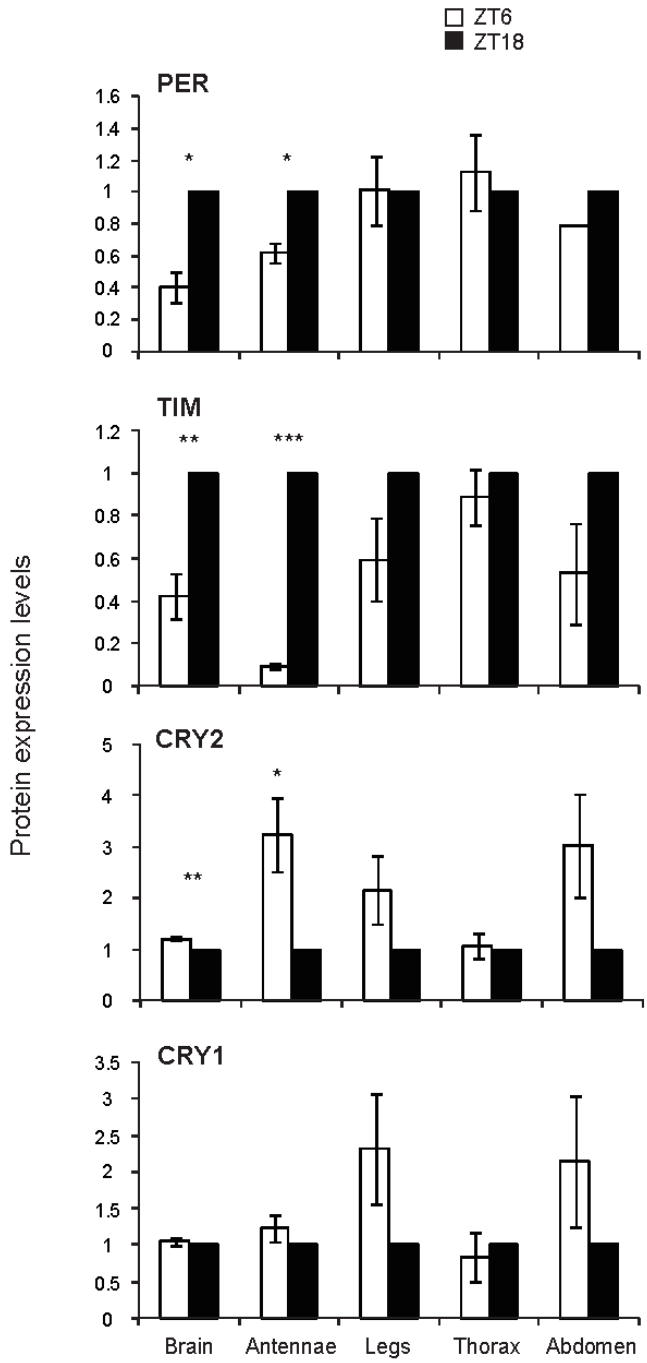

Figure S3. Temporal patterns of clock protein abundance in various tissues of monarch butterflies. Samples were collected, treated and normalized as in Figure 2A and C excepted that the value at ZT6 was normalized to the level of expression at ZT18 within each tissue and represents the mean ± SEM of 5 animals, excepted for the abdomen. In this tissue, values for TIM, CRY2 and CRY1 expression levels represent the means ± SEM of 3 animals and PER expression represents data from a single animal. White bars, ZT6; black bars, ZT18. *p*-values, Student's t-test. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

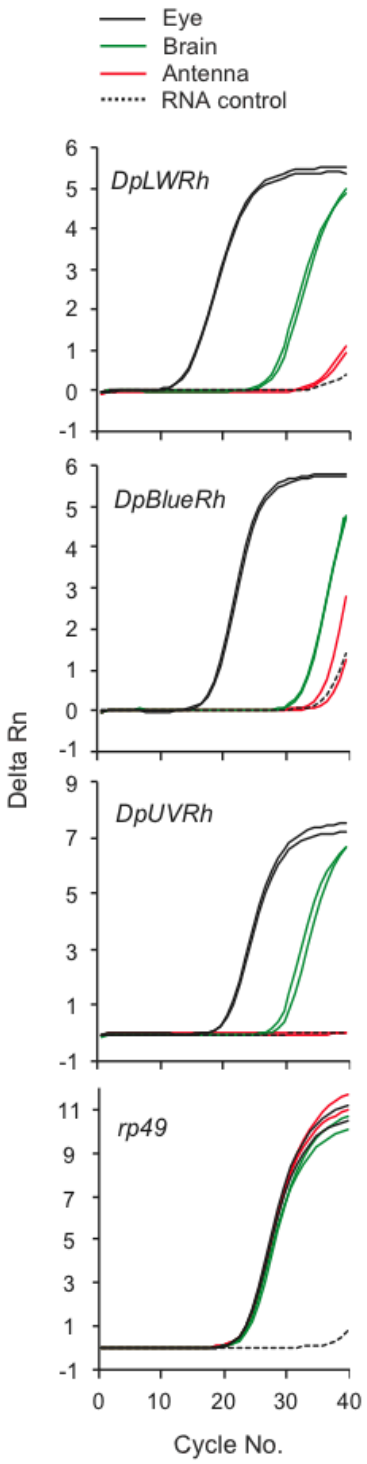

Figure S4. Quantitative real-time PCR (qPCR) detection of opsin genes expression in the antennae. qPCR amplification curves using blue opsin (*DpBlueRh*), long wavelength opsin (*DpLWRh*), UV opsin (*DpUVRh*) and *rp49* (standard) detectors on mRNA obtained from the eye (black), the brain (green) and the antenna (red) of two butterflies. Solid lines: cDNA; dotted line: DNase treated RNA control. Delta Rn is the change in fluorescence from the previous cycle.

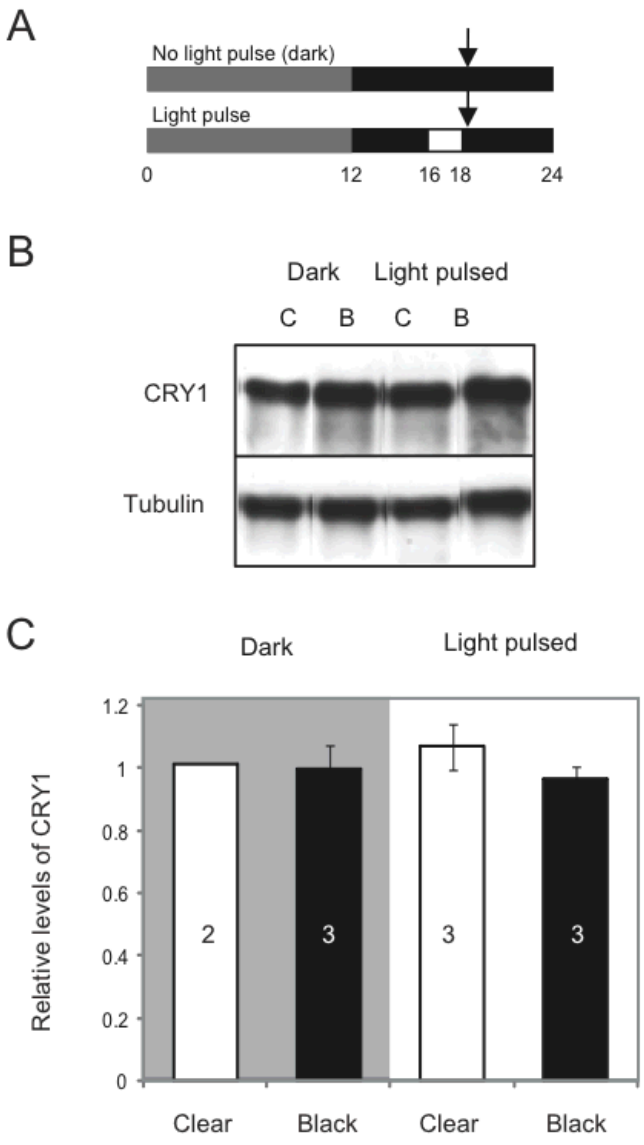

Figure S5. A light-induced decrease in CRY1 is not essential for a light-induced decrease in TIM abundance in antennae. (A) Paradigm for light-pulse study; arrows indicate the collection time. (B,C) Effects of light pulse on CRY1 levels in antennae painted clear or black. Protein levels were determined by western blots (with GP37) as in Figure 3. (B) A representative blot of the samples pooled. (C) Quantification of CRY1 levels in single painted antennae. Each value is the mean ± SEM of 3 antennae, excepted for the clear-painted antennae in dark control condition for which only the mean of 2 antennae is shown.

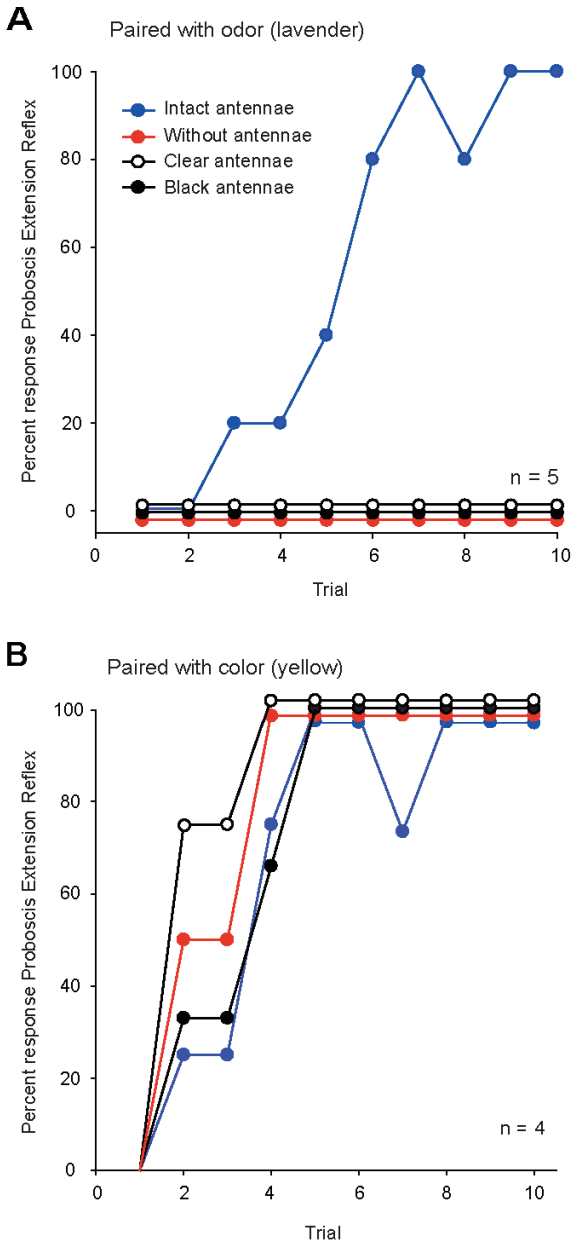

Figure S6. Painting the antennae abolishes olfactory responses. (A) Associative olfactory conditioning of the proboscis extension reflex of butterflies with intact antennae (blue line), without antennae (red line), with antennae painted clear (black line, open circles) or with antennae painted black (black line, plain black circles) along 10 training trials. Butterflies were trained with paired presentations of a lavender odor and a reward of sucrose solution. (B) Associative visual conditioning. Butterflies were trained with paired presentations of the color yellow and a reward of sucrose solution. Groups as in (A).

Results: Intact monarch butterflies with unpainted antennae exhibited robust associative olfactory conditioning, using the proboscis extension reflex with paired presentations of a lavender odor with sucrose reward, while antennae-less butterflies and those with both antennae painted with either clear or black paint did not exhibit associative olfactory conditioning.

The olfactory defect in migrants with painted antennae was a true lack of odor responsiveness, and not a defect in learning needed to perform the task, because butterflies with painted antennae (either clear or black) exhibited associate visual conditioning, using the color yellow, as easily as those with unpainted antennae.

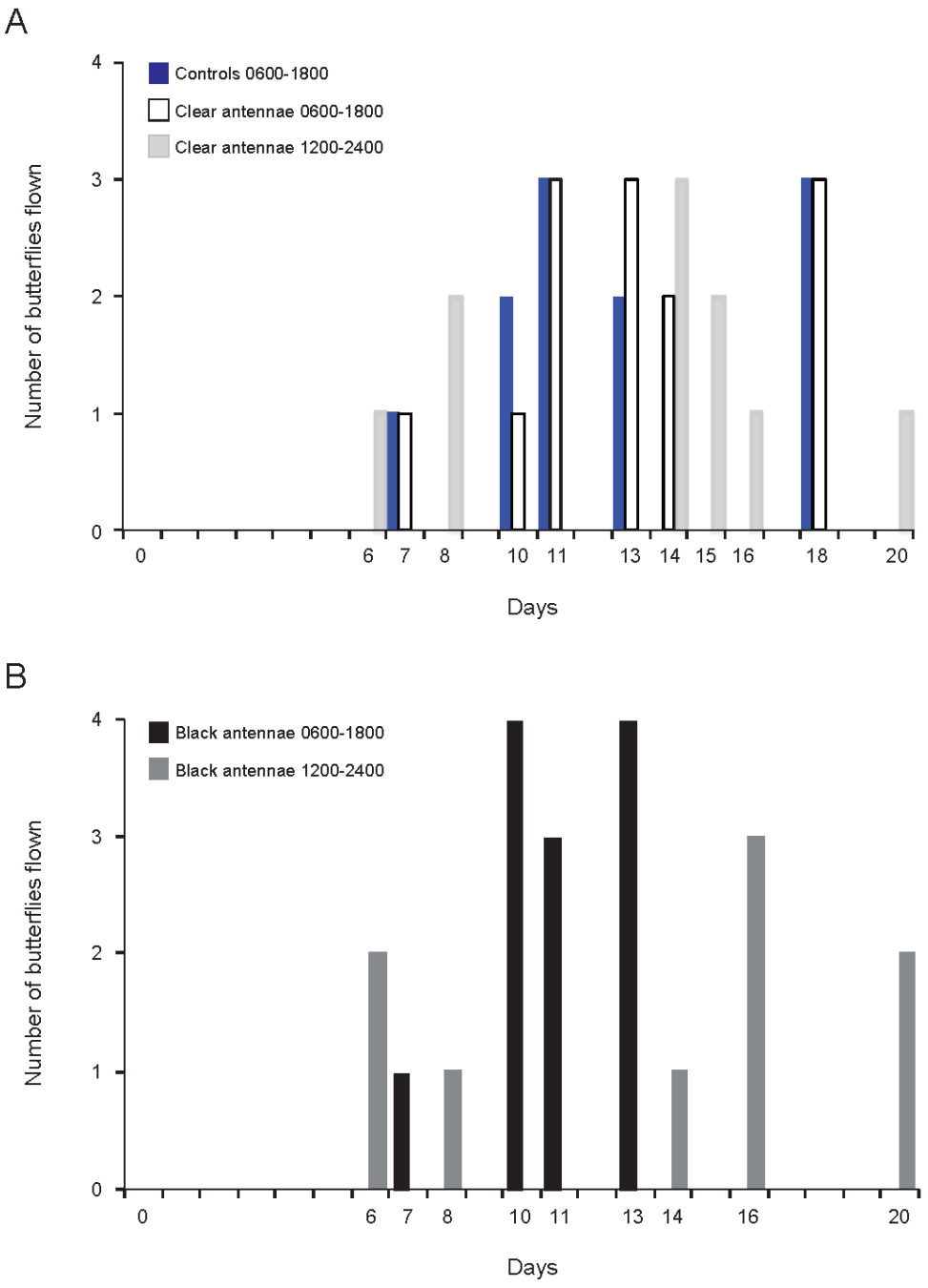

Figure S7. Temporal distribution of migrant butterflies with painted antennae flown over the course of study. Day 0 corresponds to the time of painting and the beginning of entrainment to two different LD conditions (lights on from 0600 to 1800 hours or lights on from 1200 to 2400 hours). (A) Control non-treated migrants and migrants with clear-painted antennae entrained in either of the two the different LD cycles. (B) Migrants with black-painted antennae entrained in either of the two different LD cycles. In both panels, the gap between day 0 and day 6 corresponds to the time necessary for entrainment in each lighting condition.

Supporting References and Notes

S1. H. Mouritsen, B. J. Frost, *Proc Natl Acad Sci U S A* **99**, 10162 (2002).
S2. O. Froy, A. L. Gotter, A. L. Casselman, S. M. Reppert, *Science* **300**, 1303 (2003).
S3. S. M. Reppert, H. Zhu, R. H. White, *Curr Biol* **14**, 155 (2004).
S4. H. Zhu, R. J. Gegear, A. Casselman, S. Kanginakudru, S. M. Reppert, *BMC Biol* **7**, 14 (2009).
S5. E. Batschelet, *Circular statistics in biology* (New York: Academic Press XVI, London, 1981), pp. 371.
S6. H. Zhu *et al.*, *PLoS Biol* **6**, e4 (2008).