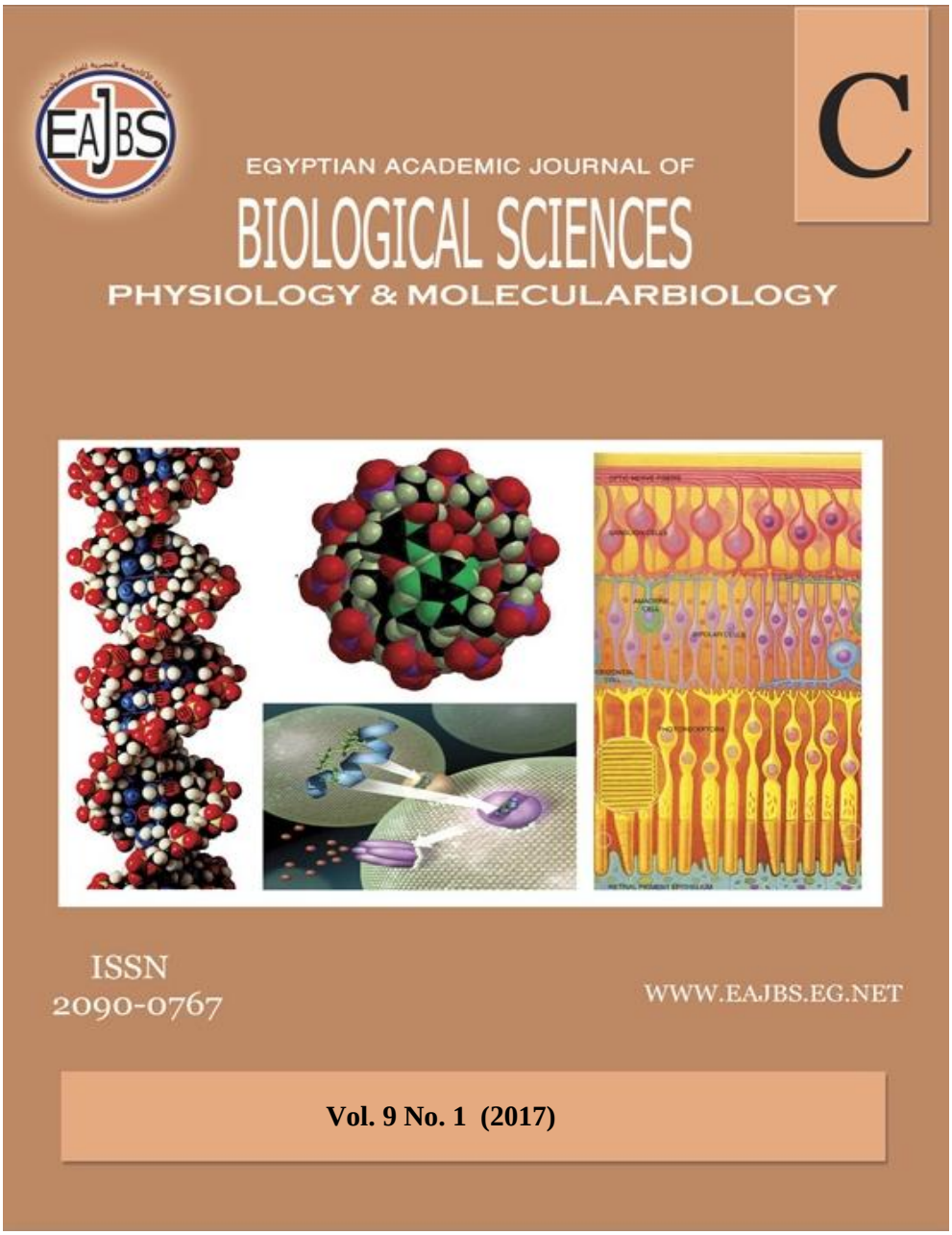

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Altered CRYPTOCHROME Expression Profile is Correlated with Reduced Circadian Clock Plasticity in the *yellow white, Drosophila melanogaster*

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ABSTRACT

The circadian clock that regulates behavior and physiology of animals is synchronized to the natural, 24 hour, light-dark cycle primarily by light. In *Drosophila melanogaster*, light is transduced to the clock neurons via the external photoreceptors in addition to the blue-light photopigment, CRYPTOCHROME (CRY), which plays a special role in clock phase resetting. Previous studies showed that the *y w* mutation in *D. melanogaster* is correlated with altered locomotor and molecular circadian rhythms, probably due to their disturbed eye pigmentation and sensitivity, and neurotransmitter physiology. This study investigates the relationship between this mutation and the CRY expression profile as a primary player in the photic entrainment pathway. Results revealed that CRY levels was most of the time higher in *y w* mutants compared to the wild-type *CS* fruit flies in all clock neuronal clusters expressing CRY. The reduced and delayed degradation of CRY indicates week photoreception by the mutants and provides a possible explanation for the delayed PERIOD (PER) and suppressed Pigment-Dispersing Factor (PDF) signaling detected previously. It could also explain the delayed morning and advanced evening locomotor activity peaks of these *y w* mutants. The results also showed a higher level of variation in CRY staining between individual neurons within each cluster, probably due to reduced PDF signaling which is known to internally synchronize clock neurons. It could be concluded that, the mutations affecting the photic entrainment pathway to the circadian clock, like the *y w* mutation, would probably have widespread effects on the circadian clock machinery and the overt rhythms under its regulation

INTRODUCTION

Circadian clocks help organisms anticipate and prepare for daily rhythmic environmental changes by modulating their behavior and physiology. Light is the primary and most reliable entraining environmental cue for the circadian clock in most animals. It synchronizes the phase of the endogenous clock to the correct phase of the natural light-dark cycle, so that the overt rhythms of behavior and physiology occur at the appropriate time of day. In the fruit fly, *Drosophila melanogaster*, the brain contains about 150 neurons that rhythmically express clock genes and proteins. These neurons are known as “clock neurons” and are divided into distinct ventral and dorsal clusters. To synchronize correctly with the natural light-dark cycle, light is transduced to these neurons through three independent input pathways; the compound eyes and ocelli, the extra-retinal Hofbauer-Buchner (H-B) eyelets, and the protein photopigment CRYPTOCHROME (CRY) (Emery *et al.*, 1998; Stanewsky *et al.*, 1998).

Many of the clock neurons express CRY, which is sensitive to UV and blue light, and thus enable clock neurons deep in the brain to directly perceive light (Yoshii *et al.*, 2015).

Light received by the H-B eyelets projects to the large cluster of ventrolateral neurons (l-vLNs) (Shang *et al.*, 2008), which in turn project throughout the optic lobe and especially in the vicinity of the small cluster of ventrolateral neurons (s-vLNs) (Helfrich-Förster *et al.*, 2007) where it mediates clock phase resetting at dawn (Sheeba *et al.*, 2010). Light input to clock neurons induces severe degradation of TIMELESS (TIM) levels in all clock neurons within 30 minutes, consequently destabilizing PERIOD (PER) and resetting the phase of the core clock (Hardin, 2011). CRY is also degraded after activation by light, but more slowly than TIM degradation (Busza *et al.*, 2004; Peschel *et al.*, 2009). Studying *Drosophila* CRY mutants revealed its functions. For example, cry^{baby} (cry^{b}) show failure in light entrainment (Stanewsky *et al.*, 1998), while cry^{0}, are less sensitive to light pulses and exhibit reduced phase responses (Kistenpfennig *et al.*, 2012).

The *yellow white* (*y w*) mutation in *D. melanogaster* causes eye pigmentation loss, impaired vision, disturbed neurotransmitters levels and distributions (Summers *et al.*, 1982; Biessmann, 1985; Ewart and Howells, 1998). These flies exhibit disturbed neural control of various behaviors including abnormal male sexual behavior (Krstic *et al.*, 2013), in addition to reduced plasticity of circadian locomotor rhythms (Hassaneen, 2015) and altered circadian oscillation of PERIOD (PDF) and the Pigment-Dispersing Factor (PDF) (Hassaneen, 2017). This study aims to investigate the effect of the *yellow white* mutation on the CRY expression rhythms in the photic-entrainment and light input pathway to the circadian clock in the fruit flies *D. melanogaster*. Results are expected to provide insights for better explanation of behaviors studied in the *y w* genetic background upon integration of the circadian regulation parameters.

MATERIALS AND METHODS

Experimental animals

Adult male *D. melanogaster* flies at the age of 4-7 days after eclosion were used in the experiments. The wild-type strain *Canton S* (*CS*) was used as control against the *yellow white* (*y w*) mutant *D. melanogaster*. Both were obtained from the University of California San Diego *Drosophila* Species Stock Center (DSSC). All flies were reared on standard cornmeal/agar medium with yeast at 18°C and a cycle of 12 hours light – 12 hours dark (LD 12:12).

Antibodies

PDF staining was carried out to pinpoint the large (l-vLNs) and small (s-vLNs) subsets of the ventro-lateral clock neurons, which are PDF^{+}. A commercially available monoclonal anti PDF serum, obtained from the (Developmental Studies Hybridoma Bank at the University of Iowa) was used to stain PDF. The antibody was raised by immunizing balb/c mice with the amidated *Drosophila* PDF peptide (NSELINSLLSLPKNMNDA-NH2) by Pick Cell Laboratories B.V. (Amsterdam, The Netherlands) (Cyran *et al.*, 2005). The antibody reliably labels only PDF^{+} neurons in adults and larvae. No staining is observed in Pdf^{01} mutants. For CRY staining, a polyclonal antiserum against *D. melanogaster* CRY (dCRY) was generated by immunizing rabbits with a full-length protein fused to a histidine tag and purified from *Escherichia coli* extracts (Yoshii *et al.*, 2008). The specificity of the antibody was proofed using the *cry* null mutant (cry^{out}) in which there was no detection of specific signals in immunostainings of these flies.

A fluorescence-conjugated Alexa Fluor antibodies of 635 nm (goat anti-mouse) and 488 nm (goat anti-rabbit) were used as secondary antibodies (Molecular Probes, Carlsbad, CA).

Immunohistochemistry

Adult male brains were used for immunostaining. Brains of flies entrained to LD12:12 (500 lux) at 20°C for at least 4 days were collected every four hours at Zeitgeber times (ZT 3, ZT 7, ZT 11, ZT 15, ZT 19, ZT 23); 20 flies each. Flies were in complete dark covered with foil in all subsequent steps. Flies were fixed for 2.5 hours in 4% paraformaldehyde (PFA) in phosphate buffer (PB; 0.1 M; pH 7.4) with 0.1% Triton-X-100 on a shaker. After fixation, the flies were washed three times in PB for 15 minutes and were dissected in PB. 5% normal goat serum (NGS) in PB with 0.5% Triton X-100 was applied onto the brains as blocking buffer at 4°C overnight. The brains were then incubated in the primary antibody solution of anti-PDF (1:1,000) and anti-CRY (1:1,000) in PB with 5% NGS and 0.5% Triton X-100) for 48 hours at 4°C. This was followed by six washes in PB with 0.5% Triton X-100 for 10 minutes each. The brains were then incubated in secondary antibodies with a dilution of 1:200 in PB with 5% NGS and 0.5% Triton X-100 for three hours at room temperature. Brains were washed again six times in PB with 0.5% Triton X-100 for 10 minutes then were embedded in Vecta-shield medium (Vector Laboratories, Burlingame, CA) and mounted on glass slides (Hermann *et al.*, 2013).

Microscopy and image analysis

Immunofluorescent brains were analyzed using a laser scanning confocal microscope (Leica TCS SPE; Leica, Wetzlar, Germany). Confocal stacks of 2 µm thickness were recorded. Two diode laser lines were used for double (488 and 635 nm) immunolabelling that excites the fluorophores of the secondary antibody.

The concentration of CRY was quantified by measuring the staining intensity of the neurons containing the proteins. The two hemispheres of eight to 12 brains were analyzed for each ZT group. Leica Application Suite Advanced Fluorescence Lite Software (LAS AF Lite, 2.2.1 build 4842) was used to view complete confocal stacks. Cropping stacks and overlays generation was done using Fiji distribution of the Image J (http://rsb.info.nih.gov/ij), an open-source software freely available for data analysis in life sciences (Schneider *et al.*, 2012). Images were converted to grayscale and the brightness value from zero to 255 was used as a measure of staining intensity. Staining intensity of each neuron minus the average intensity of a similar-sized area from the background was calculated. Each measurement was repeated three times then averaged for each neuron. Intensity staining for each neuron of the LN subsets was measured, while 5 representatives were selected for each DN neuronal subset.

Data analysis

Data were analyzed and plotted using Microsoft Excel 2016 (Microsoft Corp., Redmond, WA) and SPSS Statistics for Windows version 22.0 (IBM Corp., Armonk, NY). Student *t*-test was used at a significance probability of ($p<0.05$).

RESULTS

CRY expression profile

In the wild-type *CS D. melanogaster*, CRY is expressed in the l-vLNs, the s-vLNs including the 5^{th} s-vLN, three neurons of the dorsal lateral neurons (dLNs), and in the first subset of the dorsal neurons (DN1s). Staining results showed that in the *y w* mutants, CRY expression pattern and locations was generally preserved (Fig. 1), however, with some level and timing variation (Fig. 2).

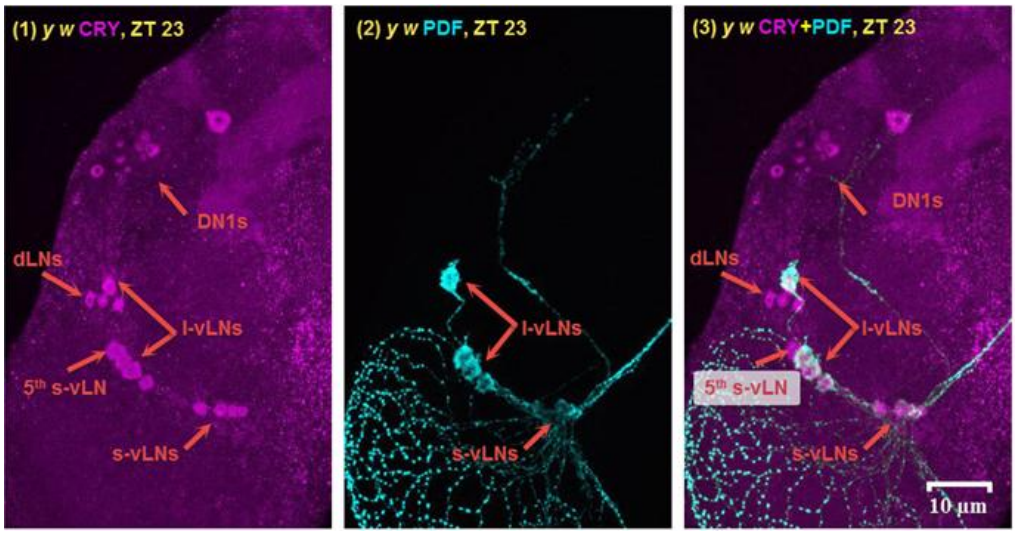

Fig. 1: Representative double immuno-labeling of CRY (Magenta) and PDF (Cyan) at the peak expression zeitgeber time (ZT 23) in the clock neuron clusters of *y w D. melanogaster*'s brains. Separated staining of CRY (1) and PDF (2) are shown as an example of single staining, while (3) is the composite double-staining of CRY+PDF. The scale bar of 10 µm shown in (3) applies to all images.

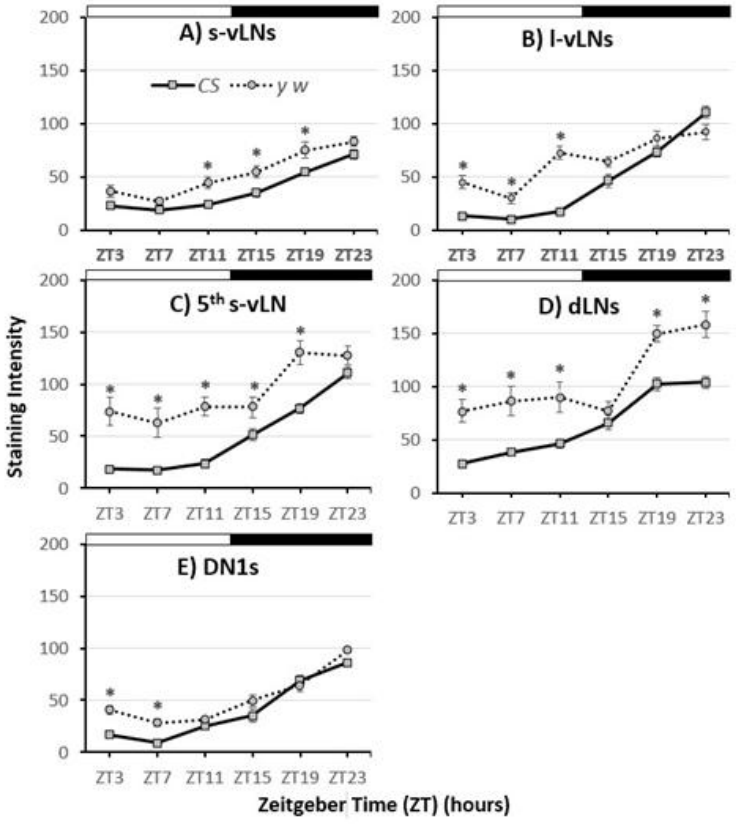

Fig. 2: Circadian profile of CRY expression in clock neuronal subsets in the *y w* and *CS D. melanogaster* brain, every four hours. Data represents the average staining intensity of neurons in the two hemispheres of 8-14 brains ± SEM. * indicates a significant difference between *y w* and *CS* at the given Zeitgeber time (ZT) using *t*-test at ($p<0.05$). Black and white bars above the figures indicate dark and light phases of the (12:12) LD cycle, where ZT 0 and ZT 12 are the lights-on and light-off times, respectively.

CRY levels, expressed in staining intensity, of *CS* and *y w*, were compared statistically using *t*-test at (p<0.05), every four hours at zeitgeber times ZT 3, 7, 11, 15, 19, 23. During daytime, CRY levels was significantly higher in *y w* than *CS* in the l-vLNs, 5th s-vLNs, dLNs, and in early and midday only in DN1s, (Figure 2(B, C, D, and E)), respectively. While, during night-time, CRY was significantly higher in *y w* than *CS* in early and mid-night in the s-vLNs and the 5th s-vLN (Figure 2(A and C)), and in mid- and late-night in the dLNs (Figure 2(D)).

Variation of CRY levels within clusters of clock neurons

Results revealed that the variation of CRY levels within each clock neurons cluster was significantly higher in *y w* mutants compared to *CS* in all clusters except in the DN1s (Fig. 3).

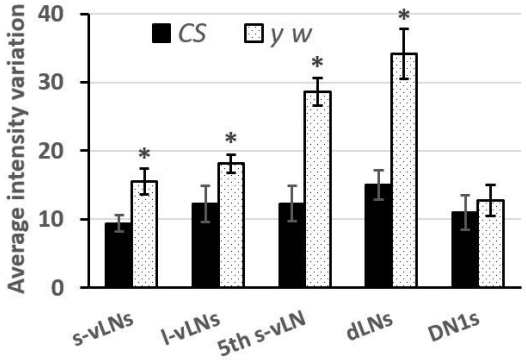

Fig. 3: Variation in staining intensity within each cluster of clock neurons in *y w* and *CS* *D. melanogaster*. * indicates a significant difference between *y w* and *CS* at a specific neuronal cluster using *t*-test at (p<0.05).

DISCUSSION

D. melanogaster flies require either CRY or the visual system to synchronize to light-dark cycles (Helfrich-Förster *et al.*, 2001; Klarsfeld *et al.*, 2004). Eliminating photic input pathways, either by loss of eyes and ocelli or disabling opsin-based photoreception by vitamin A depletion or in phototransduction mutants, reduces light sensitivity of the clock, but doesn't abolish entrainment of locomotor activity rhythms by light, which suggests the presence of other photoreceptors (Hardin, 2011). Screening rhythm mutants identified CRY as a potential photoreceptor (Lin *et al.*, 2001). Further studies showed that CRY is a cell-autonomous photoreceptor that resets the phase of behavioral rhythms in response to short pulses of light, but is not essential for entrainment to LD cycles (Emery *et al.*, 2000). As a blue light photoreceptor, and since the proportion of blue light increases during dawn and decreases during dusk, CRY appears more particularly suited to distinguish dawn or dusk light transitions from moonlight at night, which doesn't change spectrum over time (Bachleitner *et al.*, 2007). Hypomorphic cry^b mutants exhibit severely damped phase responses to light pulses, while CRY over-expression increases phase-response which suggests that CRY protein mediates circadian light responses (Stanewsky *et al.*, 1998). Besides photoreception, CRY plays a major role with the main players of the core clock. Upon photoreception, CRY is activated to bind TIM and initiates its degradation. The time of exposure to light dictates the level of TIM degradation. Light-induced degradation of TIM during the early evening delays the phase of the clock, while during late night, advances the phase. Exposure to light during daytime

doesn't affect the phase because TIM levels are already very low and can't be reduced any further (Naidoo *et al.*, 1999).

The *y w* mutant *D. melanogaster* flies exhibit delayed morning activity peak and advanced evening activity peak (Hassaneen, 2015), in addition to delayed PER degradation and suppressed PDF signaling (Hassaneen, 2017). Based on these manifestations, this study aimed to investigate the potentially correlated alterations in the clock's input pathway, especially regarding CRY, by studying its circadian expression in clock neurons. Although a proper CRY expression in all clock neuron clusters integrates to produce the normal wild-type circadian behavior, some clusters seem to contribute more than others do. For example, transgenetic expression of CRY only in the PDF-positive vLNs of cry^b mutants substantially rescue circadian light responses, with complete rescue when CRY is expressed in all clock neurons (Emery *et al.*, 1998).

The s-vLNs are considered the primary morning oscillator cells (Stoleru *et al.*, 2004), but also contribute to the evening activity peak (Rieger *et al.*, 2006). Results revealed that their levels slightly increased at the transition from light to dark, but not at the beginning of day (Figure 2(A)). This implies that their morning phase resetting might be more dependent on photoreceptors other than CRY. On the other hand, In the l-vLNs, CRY is necessary for shifting the behavioral phase, because knocking it there abolishes light-induced phase shifts (Tang *et al.*, 2010). Here, the significant high spike of CRY at ZT 11 provides a possible explanation for the advanced evening activity peak found before in *y w* mutants (Hassaneen, 2015). In the 5^{th} s-vLNs and the dLNs, CRY exhibited the highest levels compared to *CS*, especially at the night-time compared to other clock neurons (Figure 2(C and D)). This might be related to their function as primary evening clock oscillators (Grima *et al.*, 2004). However, high CRY levels persisted longer in the dLNs, probably reflecting higher hierarchy level in clock organization.

Results also revealed that CRY levels in DN1s were the least different in *y w* compared to *CS*, especially at night (Figure 2 (E)). This is probably because they are *cry*-independent deep brain photoreceptors suggested to be entrained indirectly via other *cry*-expressing neurons (Rieger *et al.*, 2003). DN1s were also found to be signaled via l-vLNs to promote light-induced phase delays (Benito *et al.*, 2008). It also appears that effects reaching DN1s are fading because they seemed situated in a delayed position in the clock neuronal pathway.

Furthermore, variation in CRY staining between the neurons of each cluster were higher in *y w* compared to *CS* flies in all clusters except in the DN1s (Figure 3). This variation is probably due to suppressed PDF signaling in *y w* mutants (Hassaneen, 2017), which functions to internally synchronize the phase of clock neurons (Helfrich-Förster, 2009).

CONCLUSION

It could be concluded that, the mutations affecting the photic entrainment pathway to the circadian clock, like the *y w* mutation, would probably have widespread effects on the circadian clock machinery and the overt rhythms under its regulation.

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ARABIC SUMMARY

التغير في مستويات الكريبتوكروم مرتبط بانخفاض مرونة استجابة الساعة البيولوجية في ذباب الفاكهة دروسوفيلا ميلانوجاستر الأصفر الأبيض المطفَّر

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الساعة البيولوجية المتحكمة في الكثير من سلوكيات ووظائف أعضاء معظم الحيوانات تزامن إيقاعها مع دورة الضوء-الظلام، والتي تتكرر كل ٢٤ ساعة، بواسطة الضوء بشكل أساسي. ففي ذباب الفاكهة دروسوفيلا ميلانوجاستر، تنتقل معلومات الإضاءة للخلايا العصبية للساعة البيولوجية من خلال مستقبلات الضوء الخارجية بالإضافة إلى الصبغ الضوئي الحساس للضوء الأزرق (كريبتوكروم) والذي يلعب دور أساسي في عملية إعادة ضبط طور الساعة. وقد أظهرت دراسات سابقة أن طفرة الأصفر الأبيض في ذباب الفاكهة مرتبطة بتغيرات في الروتين اليومي للنشاط الحركي والجزيئي، ربما بسبب اضطرابات أصباغ الأعين وحساسيتها واضطرابات فسيولوجية النواقل العصبية فيها. وتهدف هذه الدراسة لاستكشاف العلاقة بين هذه الطفرة والروتين اليومي لمستويات صبغ الكريبتوكروم وذلك بسبب دوره الرئيسي في مسار التزامن الضوئي للساعة. وقد أظهرت الدراسة أن مستويات الكريبتوكروم كانت أعلى دائماً في الذباب المطفَّر مقارنة بذباب الطرز البري كانتون إس في المجموعة الضابطة، وذلك في كل الخلايا العصبية للساعة البيولوجية المفرزة للكريبتوكروم. ويشير انخفاض وتأخر تفكك الكريبتوكروم إلى ضعف الاستقبال الضوئي في هذا الذباب. كما يقدم تفسيرا محتملاً لتأخر تفكك بروتين (بيريود) وكذلك كبح التواصل العصبي من خلال معامل نشر الصبغيات (بي دي إف)، والذي تم رصده في دراسات سابقة. كما يفسر أيضا تأخر نوبة النشاط الحركي الصباحي وتقدم نوبة النشاط المسائي في الذباب المطفَّر. وعلاوة على ذلك فقد أظهرت النتائج تباين كبير داخل كل مجموعة من الخلايا العصبية للساعة، الأمر الذي قد يعزى إلى انخفاض مستويات ال (بي دي إف) والذي يساعد على التزامن الداخلي لطور هذه الخلايا. وقد خلصت الدراسة إلى أن الطفرات التي تؤثر على مسار الإدخال والتزامن الضوئي للساعة البيولوجية ترتبط باحتمالات تأثيرات واسعة النطاق على مكونات الساعة البيولوجية الجزيئية والعصبية وكذلك السلوكيات ووظائف الأعضاء التي تتحكم بها.