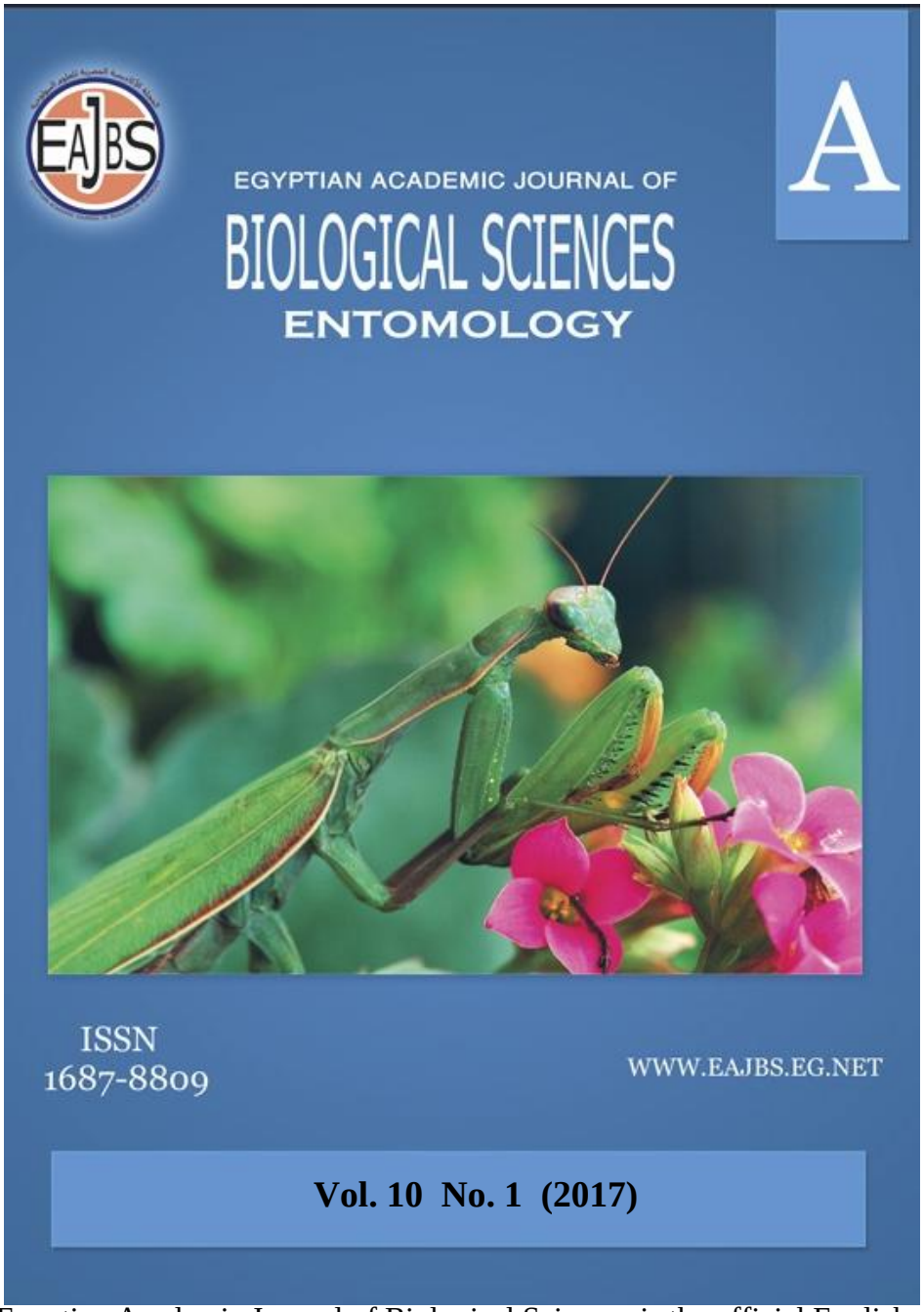

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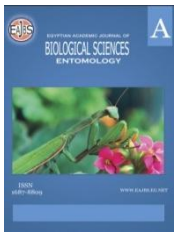

Circadian Expression of PERIOD and the Pigment-Dispersing Factor in the *yellow white* mutant *Drosophila melanogaster*

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ABSTRACT

Daily behavioral and physiological rhythms in most animals are regulated by a circadian clock. In the fruit fly, *Drosophila melanogaster*, this clock consists of a network of about 150 cerebral neurons. Different parameters of the fruit fly circadian locomotion are attributed to specific neuronal subsets and the molecular rhythms of clock genes and proteins within them. To understand the clock machinery, many clock mutant flies have been used. *yellow white* (*y w*) mutation in *D. melanogaster* cause impaired melanisation, eye pigmentation loss, and behavioral alterations including changes in circadian locomotion. This study investigates the possible molecular background for these circadian alterations. Results revealed that in the output pathway of the clock, the Pigment-Dispersing Factor (PDF) expression was suppressed in *y w* mutant fruit flies compared to *Canton S* (*CS*) wild-type in the PDF^{+} clock neurons. On the other hand, the degradation of the PERIOD (PER) protein was significantly delayed in *y w* mutants and their levels was higher, especially at the transition from dark to light. The combined effect of elevated PER levels and suppressed PDF signaling provides an explanation for the delayed morning locomotor activity peak (M) and advanced evening peak (E) of *y w* flies compared to *CS*. It could be concluded that mutations affecting eye pigmentation like the *y w* mutation could have profound effects on the circadian regulation of behavior and their underlying molecular oscillations in clock neurons. These effects reduce the plasticity and robustness of the circadian clock and expose the flies to higher levels of the environmental risk of desiccation.

INTRODUCTION

Most organisms have circadian clocks that synchronize their behavior and physiology to the daily cycling environmental conditions. The core oscillator of these clocks consists of at least three interlocked molecular feedback loops (Tomioka and Matsumoto, 2010) that occurs within clock neurons (Nitabach and Taghert, 2008). A primary loop of these involves *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), and *cycle (cyc*) clock genes (Hardin, 2006). CLK and CYC proteins produced by *Clk* and *cyc* genes form a heterodimer and bind to the E-box promoter region of *per* and *tim* to activate their transcription during late day to early night. The product proteins PER and TIM accumulate during the night to peak at late night. PER and TIM then form a heterodimer that enters the nucleus and repress their own transcription by inhibiting CLK-CYC heterodimer. Suppression of *per* and *tim* transcription leads to a reduction in PER and TIM levels, which in turn releases the suppression on CLK-CYC and eventually leads to increasing the transcription of *per* and *tim*, which starts a new

cycle (Stanewsky, 2002). The output timing signals of the clock are then relayed to overt downstream rhythms mainly via the pigment-dispersing factor (PDF), which is a peptide neurotransmitter that is considered the primary output factor for the clock.

The neuronal network of the circadian machinery are best described in *D. melanogaster* (Tomioka and Matsumoto, 2010). These clock neurons are about 150 and are divided into two major groups; the lateral neurons (LNs) located in the anterior lateral brain and the dorsal neurons (DNs) that lie in the dorsal protocerebrum (Taghert and Shafer, 2006). Each of them is further divided into three groups. The LNs are divided into: the large ventro-lateral neurons (l-vLNs), the small ventro-lateral neurons (s-vLNs) and the dorso-lateral neurons (dLNs), while the DNs are divided into DN1s, DN2s and DN3s (Helfrich-Förster, 2003). Although, it was thought for a long time that the circadian mechanism is purely cell-autonomous within these individual clock neurons, however, recently, many reports highlight the role of the neuronal interactions among multiple clock neurons of the fly brain in driving rhythmic behavior (Nitabach and Taghert, 2008; Hermann-Luibl and Helfrich-Förster, 2015).

Studying mutant *Drosophila* provided immense help in understanding the circadian clock machinery and mechanisms (Konopka and Benzer, 1971; Sehgal *et al.*, 1994; Allada *et al.*, 1998; Hardin, 2011). *yellow white* is one of these mutants. The *yellow* (*y*) gene plays a role in melanin pigmentation (Biessmann, 1985), while the *white* (*w*) gene encodes a transmembrane ABC transporter protein that is involved in the transport of vital precursors to certain *Drosophila* pigments (Ewart and Howells, 1998). The abnormal eye pigmentation in addition to reduced and altered levels of clock-related neurotransmitters have adverse effects on the behavior and circadian plasticity in these flies (Campbell and Nash, 2001; Hassaneen, 2015).

This study investigates the effect of *yellow white* mutation on the rhythmic expression profiles of PER and PDF proteins in the different clock neuronal subsets of the *D. melanogaster* brain. Results are expected to reveal the effect of possible differential alterations of clock protein expression profiles on behavioral rhythms. The study also aims to identify the specific role of each neuronal subset in regulating circadian locomotor behavior.

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

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 PickCell Laboratories B.V. (Amsterdam, The Netherlands) (Cyran *et al.*, 2005). The antibody reliably labels only PDF^{+} neurons. No staining is observed in pdf^{01} mutants. For PER protein, a polyclonal rabbit anti-PER antibody was used for staining that was raised against full-length PER expressed in a baculovirus expression system (Liu *et al.*,

1992). 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 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 in complete darkness at room temperature. 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-PER (1:6,000) 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 sequentially for double (488 and 635 nm) immunolabelling that excites the fluorophores of the secondary antibodies. The concentration of PDF and PER was quantified by measuring the staining intensity of the neurons containing the proteins. The two hemispheres of eight to twelve 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 ImageJ (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. The staining intensity of each neuron was measured minus the average intensity of a similar-sized area from the background to standardize background-staining differences. Each measurement was repeated three times then averaged for each neuron. Intensity staining for each neuron of the LN subsets was measured, while five 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 for statistical analysis at a significance probability of (p<0.05).

RESULTS

PDF expression profile

PDF is expressed only in s-vLNs and the l-vLNs. Statistical analysis using *t*-test at (p<0.05) between the PDF staining intensity of *y w* and *CS D. melanogaster* at

each Zeitgeber time tested revealed that in the s-vLNs; PDF expression level in *y w* was always significantly lower than wild-type *CS* (Figure 1 (1-3 and 10-14) and Figure 2 (A)). While, in l-vLNs, it was only significantly lower during daytime only at ZT 3, 7, 11 (Figure 1 (1-3 and 10-14) and Figure 2 (B)).

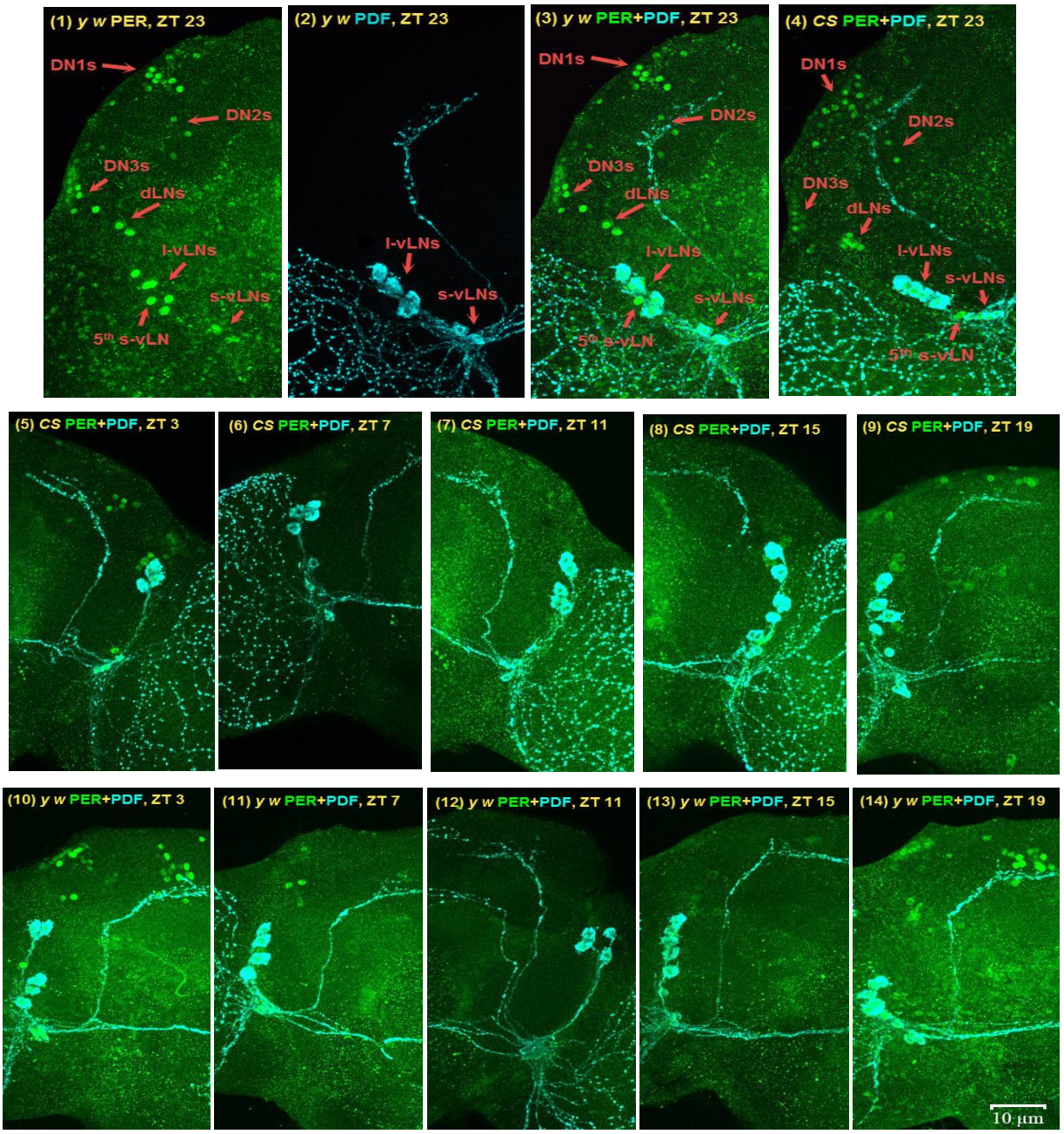

Fig.1: Double immuno-labeling of PER (Green) and PDF (Cyan) showing their circadian expression in the clock cell clusters of representative *D. melanogaster*'s brains. Separated staining of PER (1) and PDF (2) in *y w* flies at ZT 23, when cells are best visible, are shown as an example of single staining, while all other images are shown as composite double-staining of *CS* (4-9) and *y w* (3 and 10-14), every four hours. Cells are labelled in detail in (1-4) and are applicable to all images, with more description in the text. The scale bar of 10 µm shown in (14) applies to all images.

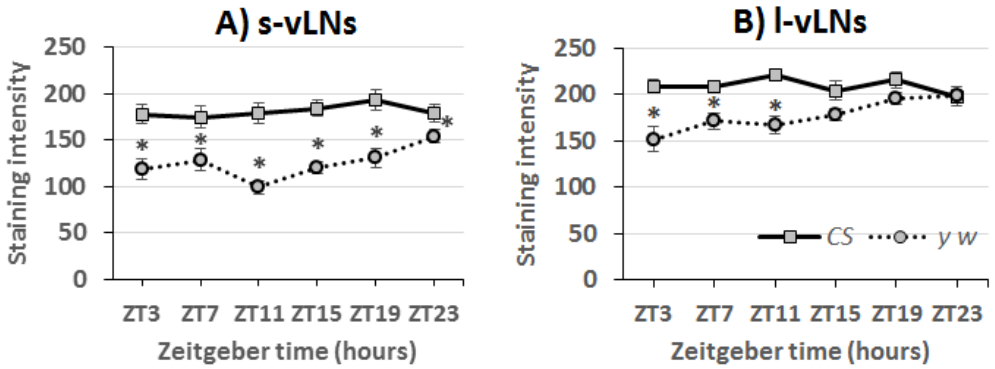

Fig. 2: PDF expression profile measured by immunostaining intensity of s-vLNs (A) and l-vLNs (B) of *y w* and *CS D. melanogaster* flies every four hours. Data represents the average staining intensity of neurons in 8-12 brains ± SEM. * indicates a significant difference between *y w* and *CS* at the given Zeitgeber time (ZT) using *t*-test at (p<0.05).

PER circadian expression

PER is expressed in all clock neurons of *D. melanogaster*. PER expression profile in *y w* mutants exhibited the general profile known in wild-type *CS*, by degrading during the light phase (ZT 3 to 11) then accumulating again during the dark phase (ZT 15 to 23) (Figure 1 and 3), however with some differences. PER staining in *y w* against *CS* at each ZT time tested were statistically analyzed using *t*-test at (p<0.05) (Figure 3).

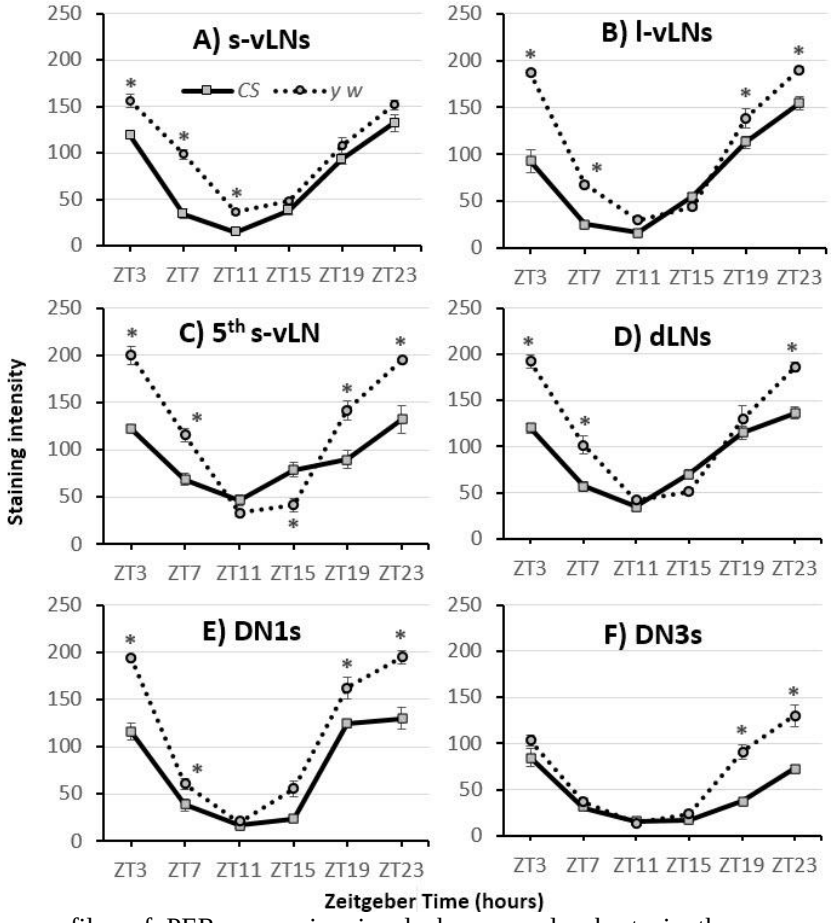

Fig. 3: Circadian profiles of PER expression in clock neuronal subsets in the *y w* and *CS D. melanogaster* brain every four hours. Data represents the average staining intensity of neurons in two hemispheres of 8-12 brains ± SEM. * indicates a significant difference between *y w* and *CS* at the given Zeitgeber time (ZT) using *t*-test at (p<0.05).

During the light phase (daytime; ZT 3-11), PER was found significantly higher in *y w* than *CS* always in s-vLNs (Figure 3(A)), while it was higher in early and midday only (ZT 3 and 7) in all other neuronal subsets (Figure 3(B-E)), except in DN3s where they were not significantly different (Figure 3(F)).

On the other hand, during the dark phase (night, ZT 15-23), PER was found significantly higher in *y w* than *CS* in mid and late night (ZT 19 and 23) in the l-vLNs, 5^{th} s-vLNs, DN1s, and DN3s (Figure 3 (B, C, E, and F)), respectively. However, in dLNs (Figure 3 (D)), PER was higher in *y w* than *CS* only at late night (ZT 23). On the contrary, in s-vLNs, PER of *y w* and *CS* were not significantly different (Figure 3 (A)), while in the 5^{th} s-vLNs, PER was significantly lower in *y w* compared to *CS* at early night (ZT 15) (Figure 3(C)).

DISCUSSION

In the natural light-dark cycle, *D. melanogaster* exhibits a bimodal crepuscular locomotor rhythmic activity, with a morning peak of activity (M) and an evening peak (E), separated by a resting siesta period (S). The various neuronal subsets of the circadian clock contribute differentially to regulate these activity bouts (Grima *et al.*, 2004). An earlier model of the clock, namely the "double-oscillator" model assumed that the s-vLNs drive (M), while the 5^{th} s-vLN and some dLNs drive (E) (Grima *et al.*, 2004; Stoleru *et al.*, 2004). However, later studies proposed that the s-vLNs (M cells) contribute to the regulation of (E), and thus suggested that their abbreviation (M) should denote "Main" rather than only "Morning" pacemaker (Rieger *et al.*, 2006; Yoshii *et al.*, 2012). A previous behavioral study of *y w* mutants revealed that they have a functional circadian clock, but their (M) was significantly delayed by about 2.5 hours, (E) was advanced by 1.7 hours, and (S) was shortened by 1.5 hours compared to wild-type *CS* (Hassaneen, 2015). The objective of this study was to investigate if these behavioral alterations are reflected on the molecular level.

Reduced PDF expression

In this study, PDF in *y w* flies was significantly reduced compared to *CS*, always in s-vLNs and during daytime only in l-vLNs (Figure 2). This result explains the advance of (E) and also the degraded morning anticipation in our previous study of *y w* flies (Hassaneen, 2015). That is because (Renn *et al.*, 1999; Grima *et al.*, 2004) found that pdf^{01} mutant flies with no PDF or flies in which v-LNs is genetically ablated, loose morning anticipation and exhibit a 2-3 hour advance of the evening activity. On the contrary, injecting the related pigment-dispersing hormone produced phase delays in cockroaches (Petri and Stengl, 1997), which is similar to the natural role of PDF in delaying (E) in the wild-type *CS*. Since the s-vLNs are primary circadian control neurons, the behavioral changes in *y w* flies and their reflected molecular alterations, are expected to affect them, which was manifested by reduced PDF at all time points. However, in l-vLNs, PDF reductions occurred only in daytime (Figure 3 (B)), probably because the l-vLNs are not essential for circadian control, but rather suggested to perceive light input and communicate that to the dLNs and consequently determining the phase of the evening peak (Potdar and Vasu, 2012), through their massive innervation to the optic medulla (Taghert and Shafer, 2006). Their involvement with photic input may be the reason why PDF decreased in them during daytime but not at night.

Circadian PER expression

Results showed that all neuronal subsets in *y w* flies exhibited elevated PER levels compared to *CS* at the transitions from dark to light (ZT 3 and 23) (Figure 3),

except the s-vLNs that didn't change significantly from wild-type at ZT 23, probably because they are more involved in precise determination of (M). To interpret that, we have to recall two things. First, in *D. melanogaster*, PER increase is correlated with rest phase, because PER accumulates during the night phase when the flies are resting. Second, the circadian clock was found to be highly light-sensitive to dim light transitions at dawn and dusk and this is very important for morning arousal, also, exposure to bright light during early night (dusk) delays the phase of the clock, while during late night (dawn) advances it (Bachleitner *et al.*, 2007). Furthermore, recent studies have revealed that even human circadian clocks are more sensitive at dawn time, since simulations of low light intensities at dawn phase advanced both melatonin and activity rhythms (Danilenko *et al.*, 2000; Danilenko *et al.*, 2000). Since *y w* mutants have impaired vision due to disturbed eye pigmentation (Krstic *et al.*, 2013), they seem unable to receive enough light for the morning arousal, consequently they sleep longer and the PER degradation is delayed and remains elevated during early morning compared to *CS*. Collectively these changes result in delay of (M), advance of (E) and shortening of (S).

A similar shift of activity was observed in nocturnal *Eulemur fulvus albifrons* (White-fronted lemurs) to be more day-active when they were subjected to reduced nocturnal illumination (Erkert and Cramer, 2006). Also, nocturnal *Mus musculus* mice shifted their activity towards diurnality after mutations, genetic manipulations and brain lesions that interfered with photoreception to the circadian clock (Mrosovsky and Hattar, 2005). On the other hand, natural prolonged light exposure in the summer advances the morning activity of flies (speed up) to the beginning of the day and delays the evening activity (slow down) to the end of the day to avoid the midday heat (Majercak *et al.*, 1999; Rieger *et al.*, 2006) which is important for survival, even in humans (Foster and Roenneberg, 2008).

The 5^{th} s-vLN and the dLNs are the (E) pacemaker cells (Grima *et al.*, 2004; Stoleru *et al.*, 2004). They determine the phase and amplitude of (E) in LD cycles (Hermann *et al.*, 2012). In *y w* mutants, start of PER accumulation in the 5^{th} s-vLN was much delayed and is significantly lower than *CS* at ZT 15, but then quickly rises at ZT 19 (Figure 3 (C)), leading to an advanced start of (E) (Hassaneen, 2015). This can also be attributed to the reduced photic reception of *y w* flies that might erroneously translated to early night start. In *y w* dLNs, PER cycles in phase with the 5^{th} s-vLN, however their non-significant PER decrease at ZT 15 and increase at ZT 19 compared to *CS*, might indicate a higher level for the 5^{th} s-vLN compared to dLNs in the hierarchy of the circadian control.

The DNs receive circadian phase information from the s-vLNs and there is a seasonally dependent phase difference between them (Menegazzi *et al.*, 2013). They both have fiber projections to the same area in the dorsal protocerebrum, that is suggested to be a locus for coupling between different subsets of clock neurons (Kouji and Meinertzhagen, 2009). Especially that G-protein coupled PDF receptors has been localized in neurons in the DN1s and DN3s (Lin *et al.*, 2004). However, DNs function is not yet fully understood. In this study, PER expression in DNs is advanced in *y w* mutants compared to *CS* at the transitions from dark to light at (ZT 3 and 23) in DN1s and at ZT 23 in DN3s (Figure 3(E and F)), respectively. This might be due to reduced PDF expression and results in advance of (E). These results are similar to the (E) phase advance in the pdf^{01} mutants (Renn *et al.*, 1999).

CONCLUSION

The behavioral changes in *y w* mutant *D. melanogaster* flies are reflected on the molecular oscillations of their circadian clock machinery. These changes reduce the plasticity and robustness of their circadian clock and expose these flies to higher levels of environmental risk, especially, desiccation by the high temperature of midday.

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ARABIC SUMMERY

الروتين اليومي لبروتينات (بيريود) و(عامل نشر الصبغيات) في ذباب الفاكهة دروسوفيلا ميلانوجاستر (الأصفر الأبيض) المطفَّر

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تنظم الساعة البيولوجية الروتين اليومي للأنشطة السلوكية والفسيولوجية في معظم الكائنات. في ذبابة الفاكهة، دروسوفيلا ميلانوجاستر، تتكون الساعة البيولوجية من شبكة من الخلايا العصبية يقدر عددها بحوالي ١٥٠ خلية مخية تنقسم لعدة مجموعات متمايزة. وتعزي الخصائص المتعددة للروتين اليومي للنشاط الحركي في هذا الذباب لمجموعات بعينها من هذه الخلايا العصبية وكذلك للروتين اليومي لمستويات جينات وبروتينات الساعة البيولوجية فيها. ولفهم آلية عمل الساعة البيولوجية يُستخدم ذباب مطفّر لخواص الساعة. وطفرة (الأصفر الأبيض) في ذباب الفاكهة تؤدي لاضطراب في صبغيات العين وتغيرات سلوكية تشمل الروتين اليومي للسلوك الحركي. وتهدف هذه الدراسة إلى استكشاف الخلفية الجزيئية المحتملة لهذه التغيرات في الروتين الحركي. وقد أظهرت النتائج أنه في مسار خرج الساعة البيولوجية، فإن مستويات معامل نشر الصبغيات (بي دي إف) تم تثبيطها في الذباب المطفّر مقارنة بذباب الطرز البري كانتون إس وذلك في كل خلايا الساعة المنتجة للـ (بي دي إف). من ناحية أخرى، فقد تأخر تحلل بروتين (بيريود) في الذباب المطفّر وظلت مستوياته مرتفعة وخاصة في فترات الانتقال من الإظلام للإضاءة (من النهار إلى الليل). وقد وفر التأثير المتضافر لارتفاع (بيريود) وتثبيط (بي دي إف) تفسيراً محتملاً لتأخر فترة النشاط النهاري وتقدم فترة النشاط الليلي في الذباب المطفّر مقارنة بذباب الطرز البري. وقد خلصت الدراسة إلى أن الطفرات التي تؤثر على صبغيات العين، مثل طفرة الأصفر الأبيض، لها تأثير كبير على تنظيم الروتين اليومي للنشاط الحركي وهذا التأثير له خلفية جزيئية في الخلايا العصبية المسئولة عن ذلك. وقد أدى هذا التأثير إلى انخفاض مرونة وكفاءة الساعة البيولوجية بما يعرض الذباب لمخاطر الجفاف لكونها أكثر نشاطا من الطرز البري خلال فترة النهار التي ترتفع فيها درجة الحرارة.