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德國蜚蠊之嗅覺行為與核心時鐘基因的關係

The Relationship Between the Core Clock Genes and Olfactory Behavior of the German Cockroach, *Blattella germanica*

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中文摘要

德國蜚蠊在台灣是一種重要的居家害蟲，過去的研究已經證明，在牠們的各種生理及行為上表現日週律動。在果蠅，日週時鐘的機制，已經從個體到分子層級有深入研究，然而在德國蜚蠊中仍舊十分欠缺研究。藉由氣味行為偏好實驗，監測雄性德國蜚蠊成蟲於光暗、全暗以及全亮的環境中對於聚集費洛蒙氣味的行為偏好，結果顯示，在不同環境下，超過 80%的德國蜚蠊偏好聚集費洛蒙的氣味，且偏好不隨時間而改變；然而，判斷速度僅在全暗的環境中表現自由律動，光暗及全亮的環境中則沒有顯著差異，這可能是由於德國蜚蠊的躲避行為或受到視覺的刺激所造成的遮蔽效應。接著於全暗環境中，以注射 ds*per*、ds*tim*、ds*cry2* 或 ds*Clk*＋ds*cyc* 的雄性德國蜚蠊成蟲進行實驗，結果顯示，注射 ds*per* 之德國蜚蠊，其 *per* 的 mRNA 表現量無法被降低，ds*tim*、ds*cry2*、ds*Clk* 或 ds*cyc* 則皆能被降低；而所有的時鐘基因皆不影響氣味偏好；ds*per* 或 ds*cry2* 處理之德國蜚蠊則未表現對於氣味判斷速度的日夜變化，顯示其生物時鐘可能無法正常運作。ds*tim* 或 ds*Clk*＋ds*cyc* 處理之德國蜚蠊判斷速度與對照組相同，維持其夜行性之天性，然而 CLK/CYC 異源二聚體在日週時鐘中扮演著重要角色，若 *Clk* 與 *cyc* 基因表現量降低時，受日週時鐘所控制的行為應該消失，但結果卻顯示其行為沒有消失，這可能說明 RNA 干擾技術，在費洛蒙氣味偏好實驗中，是存在限制與不足。

關鍵字：

德國蜚蠊 (*Blattella germanica*)、日週律動、時鐘基因、聚集費洛蒙、嗅覺偏好、RNA 干擾

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ABSTRACT

The German cockroach, *Blattella germanica*, is one of important household pests in Taiwan. Previous studies have been demonstrated that rhythmic expression of various behaviors. In *Drosophila*, circadian clock mechanism has been extensively studied from individual to molecular level. However, there are few studies in the German cockroach. The behavioral preference on aggregation pheromone monitors odor preference of male adult German cockroaches under LD, DD or LL condition. The result implied over 80% of individuals prefers aggregation pheromone and maintained at every time point of 24 hours. The time spent to make choice on aggregation pheromone performs free-running rhythm under DD condition. However, there is no rhythmicity under LD or LL condition. The light avoiding behavior or combining visual and olfactory stimulation should speed up the behavioral preference which caused a masking effect on its circadian oscillation under lighting condition. Furthermore, circadian regulation of the odor preference was demonstrated by injecting ds*per*, ds*tim*, ds*cry2* or ds*Clk*＋ds*cyc* under DD condition. Although the expression of *per* was not knocked-down by dsRNA injection, the expression of *tim*, *cry2*, *Clk* or *cyc* did show significantly reduced. The silencing effect of clock genes did not affect the preference on aggregation pheromone. In addition, ds*per* or ds*cry2* injected cockroaches did not show changes on aggregation

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pheromone choice. The result implied that the circadian clock was not working properly. The time spent to make choice on aggregation pheromone was reflecting the nocturnal nature of the German cockroach when ds*tim* or ds*Clk* + ds*cyc* was injected. Although the transcription factor in feedback loop of circadian clock, *Clk* and *cyc*, was knocked-down, the rhythmic behavior of odor preference was not demolished. Therefore, the implantation of RNAi technique on circadian regulation of odor preference has its limitation and deficiency.

Key words

The German cockroach (*Blattella germanica*), circadian rhythm, clock gene(s), aggregation pheromone, odor preference, RNA interference

doi:10.6342/NTU201704143

CONTENTS

doi:10.6342/NTU201704143

doi:10.6342/NTU201704143

LIST OF FIGURES

doi:10.6342/NTU201704143

LIST OF TABLES

doi:10.6342/NTU201704143

INTRODUCTION

Circadian rhythm is a rhythm with the period about a day and existing in all of species to adapt day and night cycle caused by the rotation of the Earth (Dunlap *et al.*, 2004). It is regulated by the endogenous circadian clock. The circadian clock can be entrained by the time signals of environment such as day and night, temperature, or food, and drive different physiology and behavior in synchrony with the daily fluctuation of environment (Saunders, 2002; Dunlap *et al.*, 2004).

There are many studies on circadian clock in insect species especially *Drosophila* which is the model species in circadian research (Helfrich-Förster, 2002; Hardin, 2006; Helfrich-Förster, 2006; Boothroyd and Young, 2008; Sheeba, 2008). The molecular mechanism of a circadian clock is involving several clock genes to form a negative feedback loop (Hardin, 2006; Sandrelli *et al.*, 2008). In the well-studied fruit fly *Drosophila melanogaster*, CLOCK (CLK)/CYCLE (CYC) heterodimer serves as a transcriptional factor for *per* and *tim* (Stanewsky, 2002; Hardin, 2006). The expression of PER and TIM in cytoplasm allows them to form a heterodimer and translocate into nucleus to act as a repressor on CLK/CYC heterodimer. This transcriptional translocational feedback loop is the main clockwork of a circadian clock (Tomioka and Matsumoto, 2010). The PER, TIM, CLK/CYC and mammalian-like CRY2 are found in

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the German cockroach and they are assumed to form the main feedback loop.

In functional genomics, RNA interference (RNAi) is a powerful and effective reverse genetic research tool. It is well-developed during the past decades. When cells are invaded with double-strand RNA (dsRNA), the same region as endogenous mRNA, it will be recognized and processed degradation to a 21 nucleotides of small interfering RNA (siRNA) (Meister and Tuschl, 2004; Ketting, 2011). This gene silencing effect is an effective tool for investigating gene function and widely used in non-model insects (Belles, 2010). Since the German cockroach is very sensitive to dsRNA injection for gene silencing (Belles, 2010), RNA interference is an effective way to find the function of clock genes.

Several studies on the German cockroach have been demonstrated that locomotion (Lin and Lee, 1996), insecticide resistance (Lin *et al.*, 2014) and anti-oxidative ability (Unpublished data) are under the regulation of circadian clock. Since odor preference accompanies behavioral approach which is regarded as a function of locomotion, it might be regulated by circadian clock. In addition, cockroaches have excellent olfaction to aid food searching and population aggregation (Tobin, 1981; Seelinger and Gagel, 1985; Smith and Getz, 1994; Lemon and Getz, 1999). Many studies are focusing on circadian regulation related to olfaction learning, odor preference on antennae, or

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chemosensory mechanism (Gadd and Raubenheimer, 2000; Durier and Rivault, 2001; Sakura and Mizunami, 2001; Page and Koelling, 2003; Watanabe *et al.*, 2003; Kwon, 2004; Decker *et al.*, 2007; Saifullah and Page, 2009; Tonoki and Davis, 2012; Garren *et al.*, 2013). The German cockroach is a gregarious species and attracted by aggregation pheromone from feces (Ledoux, 1945; Ritter and Persoons, 1975). Therefore, I design the Y-shaped maze to understand odor preference of the German cockroach is under the regulation of circadian clock, and which clock genes is responsible for odor preference.

doi:10.6342/NTU201704143

MATERIALS AND METHODS

Insects

The German cockroaches (*Blattella germanica* L.) were reared in group with water and dog chow and maintained at 28 ± 1 ℃ and a photo period of Light: Dark = 12: 12 h (LD) conditions. Several pieces of styrofoam were provided in the rearing jar for increasing shelters. Cockroaches were collected within 24 hours of emergence to determine the age and virginity was also maintained by keeping different sexes in separated transparent plastic box (Φ= 6 cm, H= 9.5 cm). In this study, male adults (10 – 15 days old) were used in all of the experiments.

Aggregation pheromone extraction

Feces of the German cockroaches were collected from the culture jars where nymphs and adults were reared. The aggregation pheromone was extracted from the collected feces with hexane (1 g of fecal materials with 5 ml of hexane). The feces with hexane were incubated at 50 ℃ for 3 hours to guarantee the complete extraction. A filter paper was used to filter out the feces remained. Then, aggregation pheromone in hexane was stored at 4 ℃ until later usage.

The behavioral preference on aggregation pheromone

The apparatus for monitoring the behavioral preference on aggregation pheromone

doi:10.6342/NTU201704143

consisted two parts (Fig 1). The Y-shaped maze was the main arena for a cockroach to choose two odor stations and a sucking pump was the power house to create two equal speed of air current as two odor source. Y-shaped maze was made of glass tube (I.D = 1.5 cm) with two 15 cm length of side arms and a 10 cm length of main arm. A glass tube (O.D= 1.6 cm, 12.5 cm length) with a tested cockroach, could be attached to the main arm for the odor preference test. Flow meters were used to maintain the air speed at 1L/min and air current passing through the odor bottle was regarded as an odor station for tested cockroach to choose. To ensure the cleanness of the air current before entering odor station, active charcoal was used to filter odor particles in the air.

The two choice experiment was carried out on the Y-shaped maze. A cockroach was released to the main stem and odor preference was determined by reaching the half length of the side arm. The quickness of choice was measured as the time spent from releasing the cockroach to reach the half length of the side arm and the speed of the choice was calculated as time spent over the traveling distance (15.5 cm). The preparation of odor station was arranged by using 2 mL of aggregation pheromone hexane mixture onto a filter paper and allowed hexane to vaporize under a fume hood. Then, the filter paper was placed in the bottle for 1 hour at 50 ℃ before making the bottle as a odor station. The control odor station was following the same preparation for

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aggregation pheromone except no feces in the hexane extraction. All the odor choice experiments were recorded with a camera Hero4 Black (GoPro, Inc., San Mateo, California, U.S.A.). All the glass tubes in Y-shaped maze would be washed with detergent and acetone after each odor choice experiment to ensure no odor residue on the apparatus.

dsRNA synthesis and RNAi treatment

In this study, 5 clock genes of the German cockroach, *period* (*per*), *timeless* (*tim*), *cryptochrome 2* (*cry2*), *Clock* (*Clk*), and *cycle* (*cyc*) were target genes for the experiment. All of the clock genes and were amplified by PCR with the forward and reverse primer (Table 1). The dsRNA from the plasmid containing *EGFP* fragment *enhanced green fluorescence protein* (*EGFP*) was used as the control. The dsRNAs were synthesized by MEGAscript T7 kit (Ambion, Grand Island, NY). The template of dsRNA synthesis was constructed by the PCR product with primers containing the T7 promoter sequence 5′-TAATACGACTC ACTATAGGG-3′. All PCR product sizes of the target genes were from 400 to 700 nucleotides. Synthesis of dsRNA was following the manufacturer's protocol, and all of the dsRNAs were stored at −20 °C until use.

Eight to twelve days old cockroaches were selected and 1 μg/μL of double-strand RNA were injected into each cockroach at Zeitgeber time (ZT) 05 and 11 under LD

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condition. Since each cockroach received two shots of dsRNA injection, the ds*Clk* + ds*cyc* treated cockroaches received double amount of dsRNA injection. The injected cockroaches were allowed to recover one day under LD condition. Then, thecockroaches were used in the behavioral preference on aggregation pheromone experiments under constant darkness (DD) condition at CT 09-10 or CT 21-22 of the next day.

Quantitative real time polymerase chain reaction (qPCR)

Total RNA was extracted from the head of male cockroaches immediately after the odor preference experiment with RNAi treatment. The samples were homogenized with 200 μL of Azol® (Arrowtec, Taipei, Taiwan), and total RNA was separated from protein and DNA by adding 40 μL of chloroform. About 100 μL of the upper layer (aqueous phase) of each sample was transferred into a new tube, and add 100 μL isopropanol to remove salt ions. After centrifuge, supernatant was removed, then, 200 μL 75% ethanol was added with DEPC treatment to separate out RNA. The sample was centrifuged again. Waiting for the RNA pellets to dry, and was dissolved in DEPC treatment double-distilled water. All the RNA samples were treated with DNase to remove remnant genomic DNA. Synthesis of complementary DNA (cDNA) with 1 μg of total RNA was performed by High Capacity cDNA Reverse Transcription kit (Applied

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Biosystems, Foster City, CA), following the manufacturer's protocol.

The design of qPCR primers sequence of the 5 core clock genes and the *elongation factor 1A* (*EF1A*) gene of the German cockroach, which was selected as the housekeeping gene, were shown in Table 2. qPCR was performed in triplicate with Fast SYBR Green Master Mix (ABI) on the StepOnePlus real-time system. Cycling conditions were 95 ℃ for 3 min, followed by 40 cycles of 95 ℃ for 30 s and 60 ℃ for 30 s. The reaction was concluded with the melt curve beginning from 60 to 95℃ in 0.5 ℃ increments at 5 second/ step.

Statistical analysis

All of the data were shown as mean ± SE and analysed by the Kruskal–Wallis rank sum test following post-test calculations. The calculation of statistical differences was analysed by R software, v.3.1.2 (R Core Team, Vienna, Austria).

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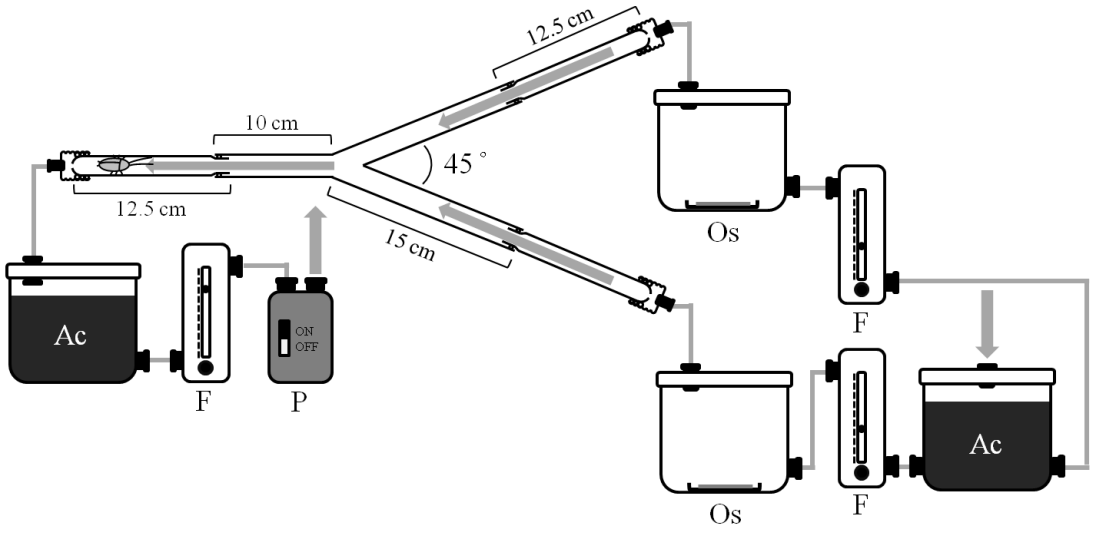

Fig. 1 The apparatus for monitoring behavioral preference on aggregation pheromone of the German cockroach. Ac: activated charcoal; F: flow meter; Os: odor station; P: suction pump. The gray arrow indicates the direction of air flow.

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Tabe 1　dsRNA primer sequenc

Gene name		Primer sequence
period	Forward	5′-GCGAACTGGTTTGGTGAGATGATG-3′
	Reverse	5′-AGGACAATCCCAGCATGTTC-3′
timeless	Forward	5′-TTGTATTGGTACTACGTTCAGAGT-3′
	Reverse	5′-GTGAATGTGCATCCTCTTGTCCTT-3′
cryptochrome 2	Forward	5′-TACAGTGGAAGCCCAGTTAACG-3′
	Reverse	5′-TAACACAGTCCGAAATACCGCA-3′
Clock	Forward	5′-TCTCACTGTCGCAGCAACAAGC-3′
	Reverse	5′-ACCATCCAAATTCCTTGAGAGGA-3′
cycle	Forward	5′-ATGATGAGGCAGCTATGGCA-3′
	Reverse	5′-AGTATCACATTATATTCCTC-3′
EGFP	Forward	5′-TATGGTGAGCAAGGGCGAGGAG-3′
	Reverse	5′-TGGCGGATCTTGAAGTTCACC-3′

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Tabe 2　qRT-PCR primer sequence

Gene name		Primer sequence
period	Forward	5′-CCCACGAAATCAAGACCAGT-3′
	Reverse	5′-TCCTCGAGCTTCAGTTTGGT-3′
timeless	Forward	5′-CCAAGAGTTGCACCATTGTCACC-3′
	Reverse	5′-CATCTCTGGACCATTCTGGCTCA-3′
cryptochrome 2	Forward	5′-GGATGCGATTGGTCGGTGATGC-3′
	Reverse	5′-CTCCATTAGGATCAGCCTTGCGT-3′
Clock	Forward	5′-ATGGCGAGATATGGCCTCCTGT-3′
	Reverse	5′-TTCCAGGCTGAGGAGGAAGTGT-3′
cycle	Forward	5′-GCAGTCCATCGCCAGAAGCAGA-3′
	Reverse	5′-GGTTCGCTGCAGTGCTGAGCTA-3′
EF1A	Forward	5′-ACCAATCTCTGGATGGCATGG-3′
	Reverse	5′-GAGGCTTCTCAGTGGGTCTG-3′

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RESULTS

Odor preference of the German cockroach

The preference of aggregation pheromone of the German cockroach was tested in a Y-shaped maze under LD, DD or LL condition. The aggregation pheromone was preferred over clean air (Fig. 2). This preference was shown in over 80% of individuals tested and maintained at every time points of the 24 hours. The preference was also not affected by the photoperiod conditions (LD, DD, or LL). The results indicated that the German cockroach displayed aggregation nature of the species whenever it alone encountered aggregation pheromone. Even through the nocturnal nature of the German cockroach, the preference of aggregation pheromone was evenly detected at various time points throughout day and night, or constant lighting/darkness (Kruskal–Wallis rank sum test, p= 0.1054, 0.1513, 0.1468 for LD, DD, LL, respectively).

The quickness of approaching aggregation pheromone was also monitored and results were shown in Fig. 3. It took about 4.09 to 8.15 second to reach the odor source. The speed of reaching odor source was different at various time points of the day, but the nocturnal nature of the species did not speed up the behavior choice under LD condition (Fig. 3A). This undifferentiated speed of approaching odor source was also detected under LL condition (Fig. 3C). However, the cockroach did show a free-running

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rhythm of approaching aggregation pheromone under DD condition (Fig. 3B). This result did reflect the behavioral approaching aggregation pheromone was under the control of circadian clock even though arrhythmic expression was found in both LD and LL condition. The conflict result may be explained by the avoid nature against light and the increasing speed of locomotion by combining visual with odor sensory.

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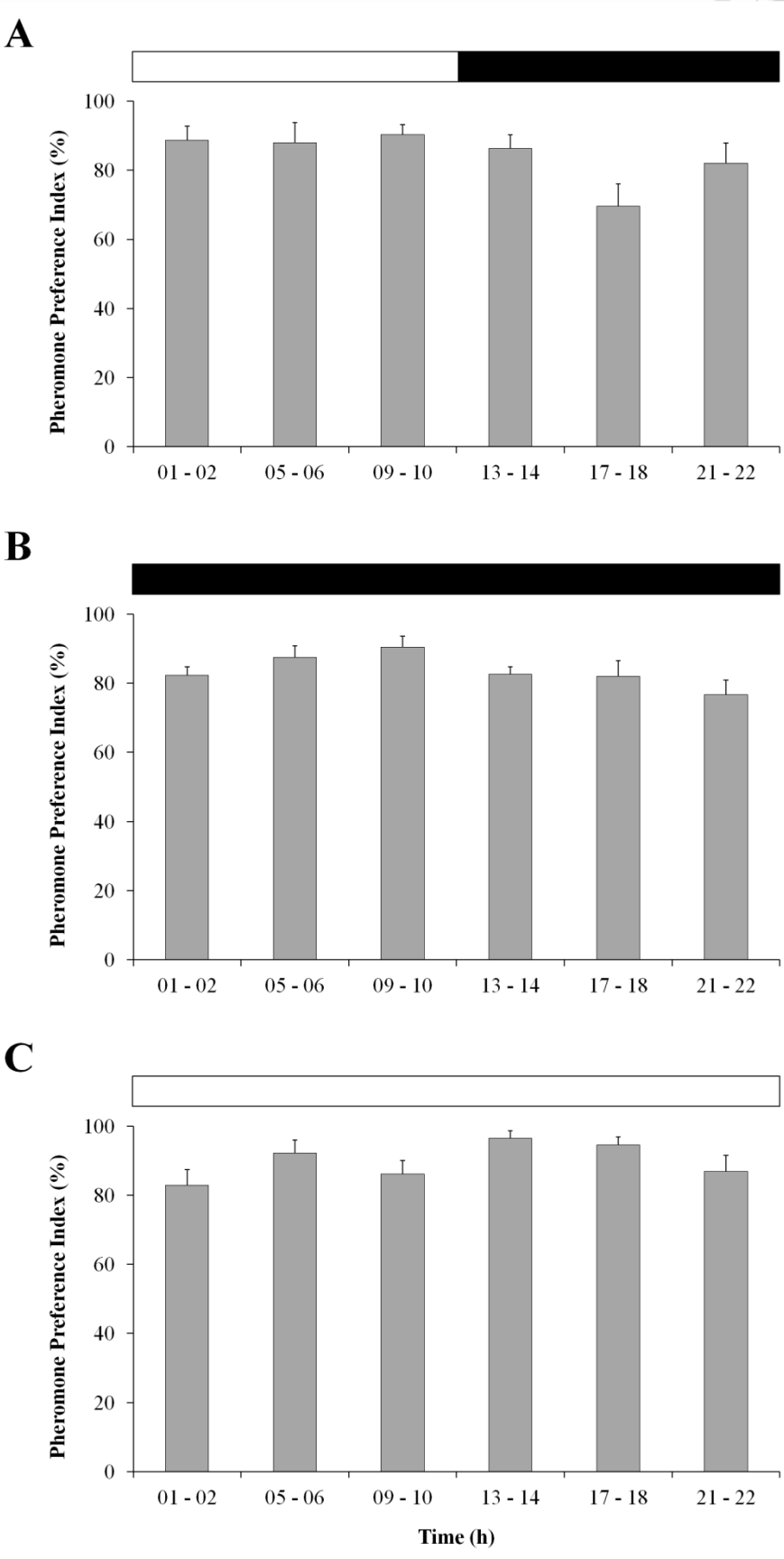

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Fig. 2 Behavioral preference of the German cockroach on aggregation pheromone in a Y-shaped maze under (A) Light-Dark = 12: 12 h (LD), (B) constant darkness (DD) or (C) constant lighting (LL). Pheromone Preference Index (PPI) was calculated as $N_P / (N_A + N_P) \times 100\%$, where N_P= number of cockroach choose pheromone station, N_A= number of cockroach choose clean air station. The error bars on the histogram indicated one set of standard error of 5 repeats ($n \geqq 48$ in total).

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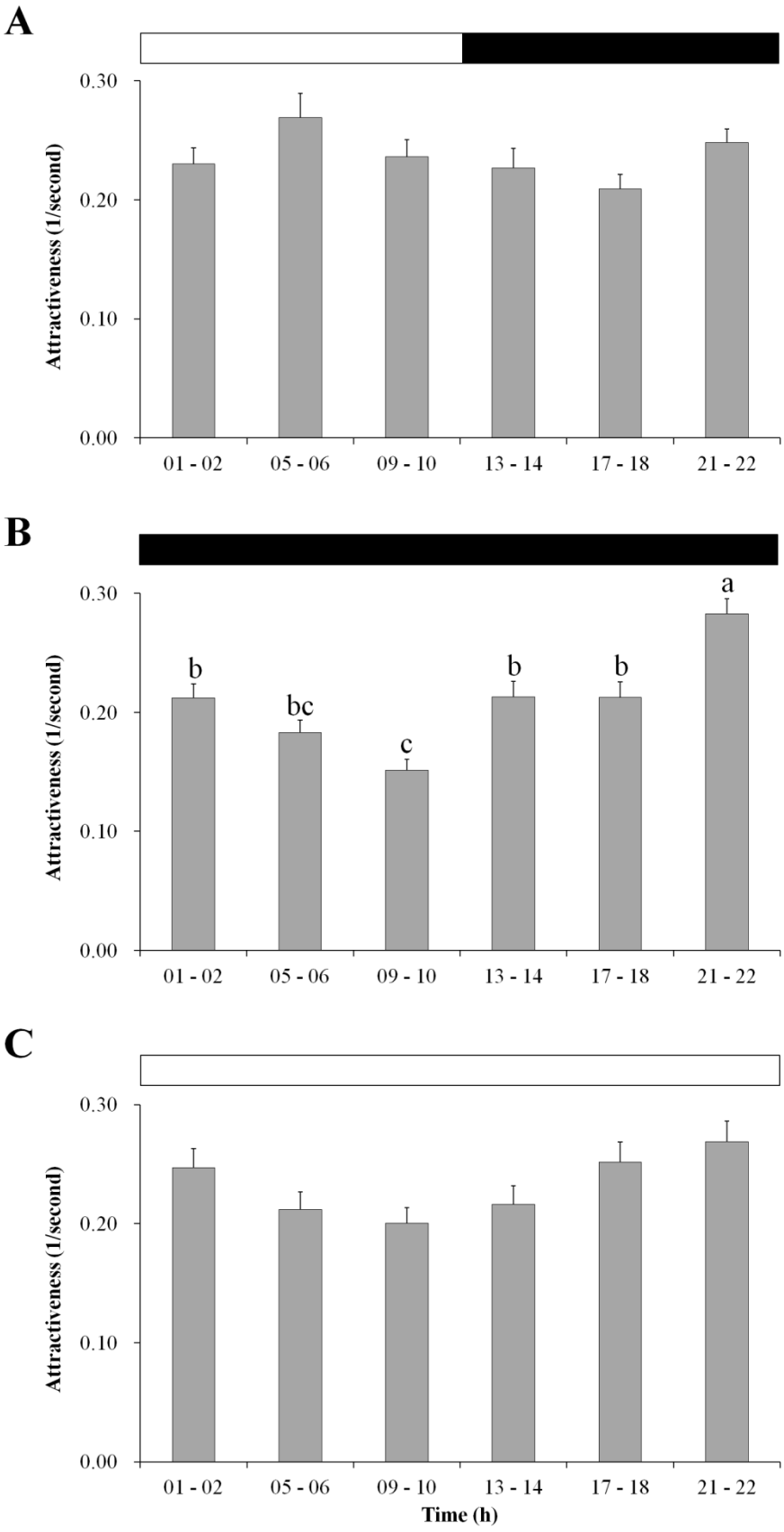

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Fig. 3 The time for behavioral preference of the German cockroach on aggregation pheromone in a Y-shaped maze under (A) Light-Dark = 12: 12 h (LD), (B) constant darkness (DD) or (C) constant lighting (LL). The reverse of time spent from the base to half length of an arm of Y-shaped maze was calculated as the attractiveness of individual cockroach by the aggregation pheromone. The error bars on the histogram indicated one set of standard error ($n \geqq 48$ in total). The different letters on the histogram indicated significant differences among various time points of the day (Kruskal–Wallis rank sum test, $p < 0.05$).

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Odor preference of RNAi treated German cockroach

The effect of double-strand RNA injection on the mRNA expression level was shown in Fig 4. The knockdown effect of RNAi on the clock genes was significant for all the genes tested except *per*. The result indicates the double-strand RNA injection did suppress the expression of the clock genes. The unique presence of secondary structure for *period* gene might imply the ineffectiveness of double-strand RNA injection (Kurreck, 2006). However, the expression level of *tim*, *cry2*, *Clk* or *cyc* at subjective day (CT 09-10) and night (CT 21-22) did show significant difference. The result might reflect the impossibility of total suppression on target gene, or the dsRNA injection could not knockdown low expression at subject day.

The effect of double-strand RNA injection on behavioral preference was shown in Fig 5. The clock genes knockdown did not affect the behavioral preference on aggregation pheromone. The comparison between subjective day and night did not show the odor preference changed according to circadian phase (Kruskal–Wallis test, p= 0.1652, 0.5025, 0.4810, 0.1047 for *per*, *tim*, *cry2*, *Clk* + *cyc*, respectively) (Fig 5) as shown in controlled cockroaches (Fig 3B). The result implies the double-strand RNA injection disrupted the operation of circadian clock to demolish the circadian rhythm expression.

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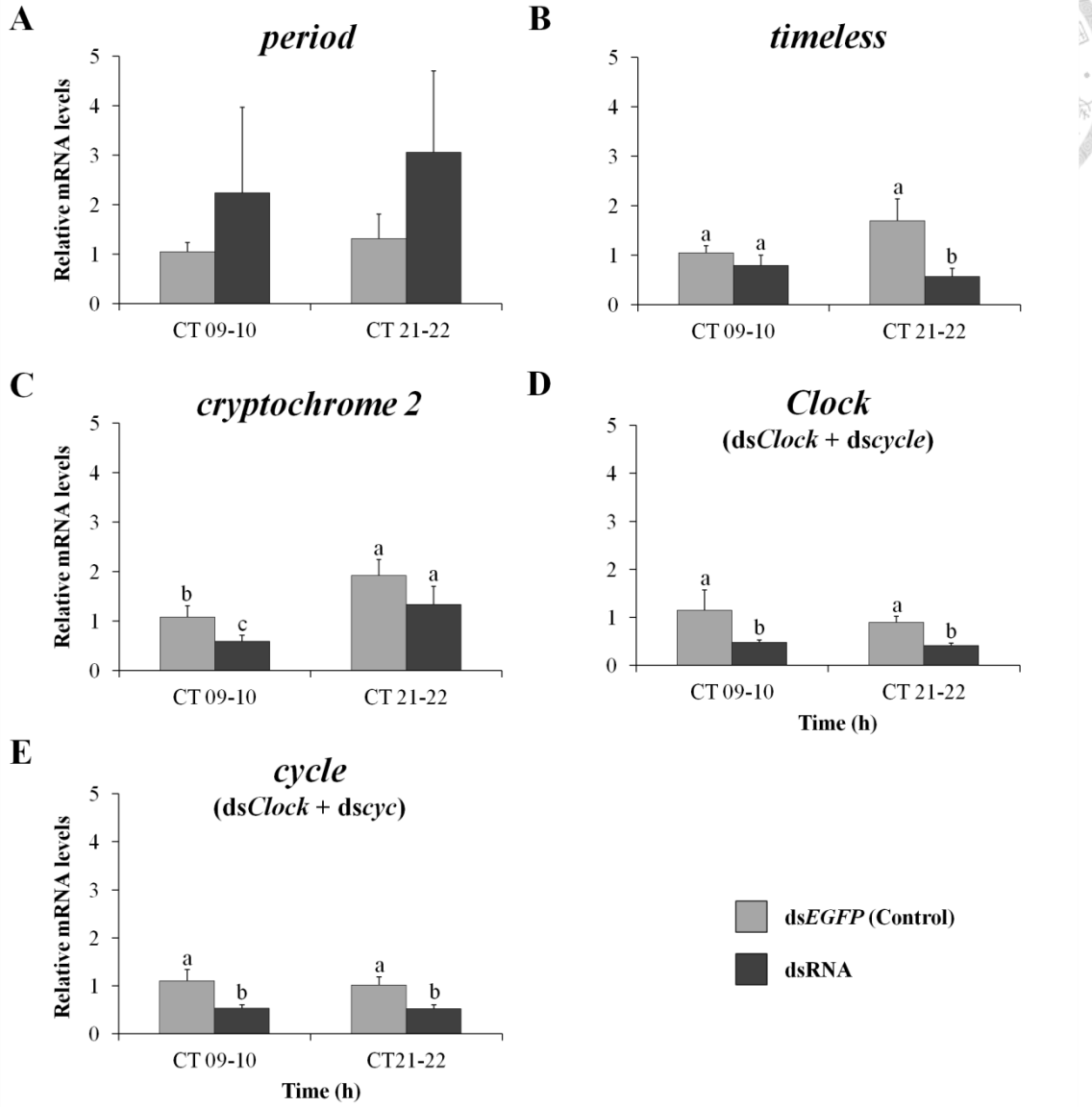

Fig. 4 The relative mRNA level of clock genes in RNAi treated German cockroach. (A) *period* (n= 16); (B) *timeless* (n≧ 11); (C) *cryptochrome 2* (n= 16); (D) *Clock* (n= 16); or (E) *cycle* (n≧ 15). The different letters on the histogram indicate significant difference at $p< 0.05$ (Kruskal–Wallis rank sum test).

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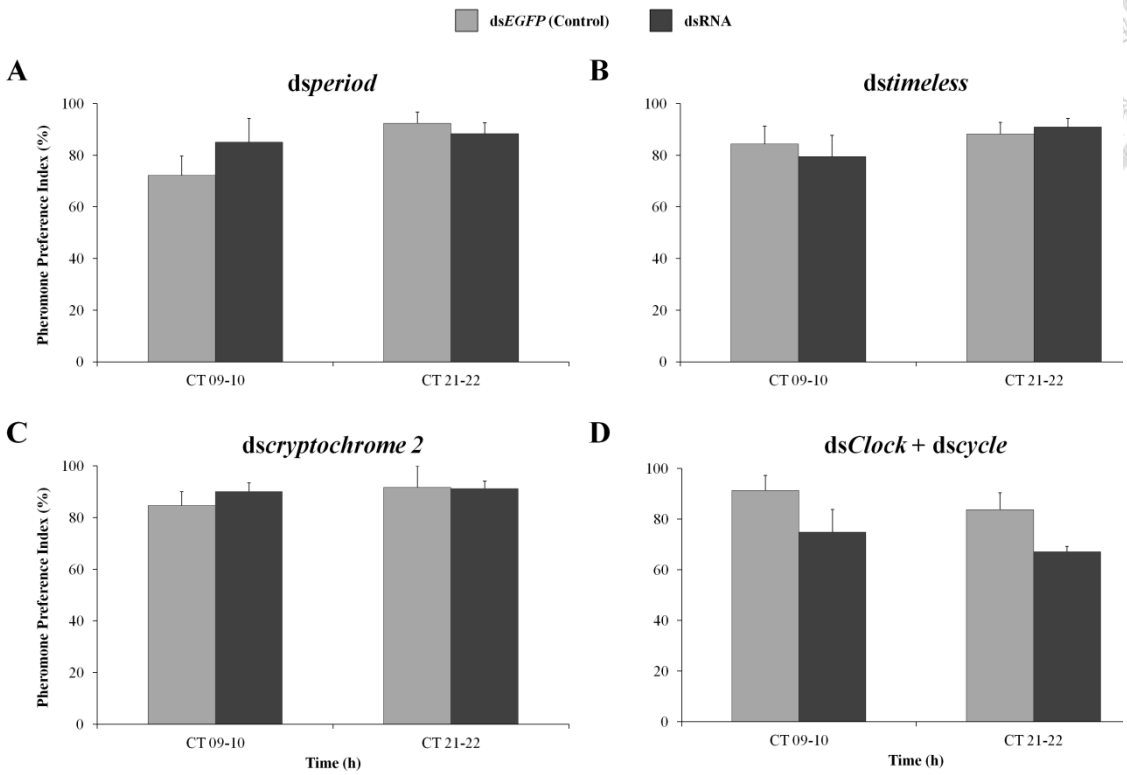

Fig. 5 Behavioral preference of double-strand (A) *period* (n≧ 21); (B) *timeless* (n≧ 30); (C) *cryptochrome 2* (n≧ 19); (D) *Clock* + *cycle* (n≧ 36) injected German cockroach on aggregation pheromone in a Y-shaped maze under constant darkness condition. There are no significant differences at $p > 0.05$ (Kruskal–Wallis rank sum test).

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The effect of double-strand RNA injection on behavioral preference of aggregation pheromone was monitored and shown in Fig.6. The ds*per* injection was the only treatment that did not cause any change on behavioral preference. It might be the failure result of RNAi treatment on *per* gene. The time spent to make choice on aggregation pheromone was reflecting the nocturnal nature of the German cockroach when ds*tim* or ds*Clk* + ds*cyc* was injected. However, ds*per* or ds*cry2* injected cockroaches did not show quicker choice on aggregation pheromone than that of control. This result implied that the circadian clock was not working properly. Since all the double-strand RNA injected cockroaches did not show knockdown effect on behavioral preference, the odor preference might not be directly reflected the circadian control. Although CLK/CYC heterodimer was the transcriptional factor for *per*, *tim*, *cry2* genes, the knockdown effect was not observed in ds*Clk* + ds*cyc* treated cockroaches. The possible explanation was the ds*Clk* + ds*cyc* injection was not strong enough to knockout the gene expression or functional CLK/CYC heterodimer was required at minimum.

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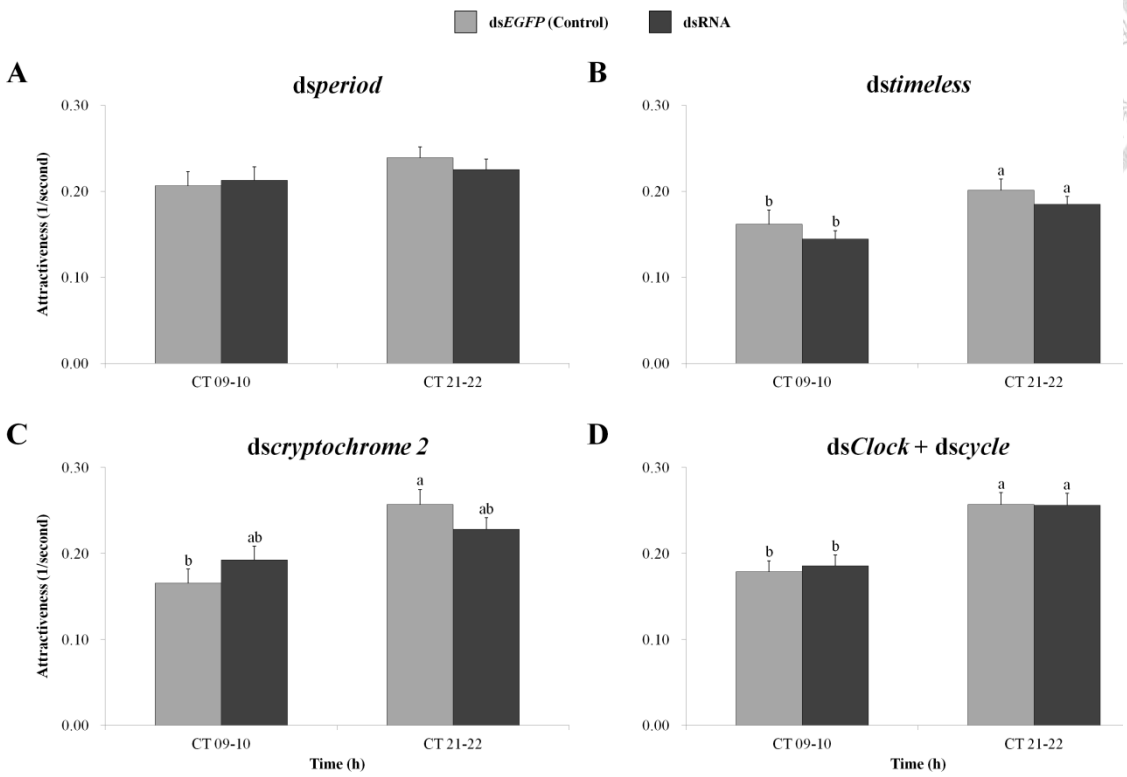

Fig. 6 The time spent to complete behavioral preference of double-strand (A) *period* (n≧ 21, p= 0.5345). (B) *timeless* (n≧ 30, p< 0.05). (C) *cryptochrome 2* (n≧ 19, p< 0.05). (D) *Clock* + *cycle* (n≧ 36, p< 0.05) injected German cockroach on aggregation pheromone preference in a Y-shaped maze (Kruskal–Wallis rank sum test).

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DISSCUSSION

The German cockroach is a nocturnal animal so that the detection of aggregation pheromone and behavioral approaching should be at its best during night time. In fact, the adult cockroaches did perform better in behavioral choice on aggregation pheromone at night (Fig. 3), even though the detection or preference was kept at the same level throughout the day (Fig. 2). This finding reveals the sensory level for odor detection and preference is not fluctuated or under the regulation of circadian clock. However, the locomotion of the German cockroach is expressing circadian rhythm (Lin and Lee, 1996), the avoiding behavior or combining visual and olfactory stimulation should speed up the behavioral preference which might mask the circadian oscillation. This masking effect was observed under LD and LL condition (Fig. 3). Therefore, the behavioral preference on aggregation pheromone of the German cockroach is under the regulation of a circadian clock. This conclusion can gain support from the olfactory learning under a circadian regulation of the Madeira cockroach (*Leucophaea maderae*) (Decker *et al.*, 2007).

Since odor preference of the German cockroach was under the regulation of circadian clock, its behavioral preference can be demolished if the clock gene is knockdown by RNAi. However, contradictory results are found in this study (Fig. 6).

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The molecular mechanism of circadian clock in the German cockroach is not completed known. The knockdown effect of one clock gene may not sufficient to alter or diminish the oscillation of the whole clock. Even the knockdown effect of known transcription factor CLK/CYC heterodimer cannot stop the oscillation. Another explanation is the expression level of clock genes may not have large amplitude different between the peak and trough so that the knockdown effect may not have a significant reduce on expression level. The third reason is the high possibility of multiple regulations on odor preference. The knockdown effect may be obscured by other regulators on the same behavior.

Several behaviors have been found to be regulated by endogenous circadian clock (Lin and Lee, 1996; Chang and Lee, 2001; Decker *et al.*, 2007; Lin *et al.*, 2014). However, the molecular clockwork may pass through a series of signal cascade to regulate the behavior or the signal of circadian clock may depend on clock control genes to express the rhythmicity. Therefore, the implantation of RNAi technique on odor preference on aggregation pheromone has its limitation and deficiency.

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