國立臺灣大學生物資源暨農學院昆蟲學系暨研究所

博士論文

Department and Graduate Institute of Entomology

College of Bio-Resources and Agriculture

National Taiwan University

Doctoral dissertation

德國蜚蠊色素散佈因子之選殖及其在日週時鐘之功能解析

Cloning and Functional Assay of *pigment dispersing factor* on the Circadian Clock in the German Cockroach, *Blattella germanica* (L.)

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中華民國 九十八 年 一 月

January, 2009

國立臺灣大學博士學位論文

口試委員會審定書

德國蜚蠊色素散佈因子之選殖及其在日週時鐘之功能解析

Cloning and functional assay of *pigment dispersing factor* on the circadian clock in the German cockroach, *Blattella germanica* (L.)

本論文係李琦玟君（D92632001）在國立臺灣大學昆蟲學系暨研究所完成之博士學位論文，於民國九十八年一月十四日承下列考試委員審查通過及口試及格，特此證明

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誌 謝

本論文能順利的完成，衷心感謝李後晶教授對我的引領與教導。老師淵博的學識及分析問題的能力讓我非常的敬佩。和老師進行討論是我最喜歡的，從每一次的討論，我能學習如何更多元也更周全的思考問題，眼界也變得寬廣。在生活上，老師對我也非常的關懷與照顧。心裡對老師的感謝很難用文字表達。老師，真的非常感謝您。

論文口試委員林金盾教授、路光暉教授，張俊哲教授、楊恩誠教授及蘇銘燦教授對論文的指正與建議，不僅使論文能更完備，也為主題的進一步研究提供了可行的方向，在此也要表達謝意。

在這段辛苦的學習過程中，要感謝許多人對我的關心與支持。謝謝系主任- 黃生主任，王震哲主任，張永達主任給我的支持，讓我能兼顧工作並完成進修。非常感謝諸亞儂老師、林金盾老師和周雪美老師在研究上給我許多支持與教導，也給我許多人生規劃上的意見及鼓勵，讓我有勇氣克服挫折繼續走下去。感謝台大昆蟲系的陳秋男教授、張俊哲教授、柯俊成教授、楊恩誠教授、蕭旭峰教授，台灣師大生命科學系的李桂禎教授、鄭湧涇教授、黃基礎教授、林陳涌教授、張永達教授、王玉麒教授、蘇銘燦教授、給我的支持與鼓勵。謝謝好朋友們- 明忠、玟娟、宜蓉、晉怡、俊祥、友信、叡陽、春美、淑美、瑞謙、錫永…的打氣，你們總讓我有好心情，能樂觀的走過這一段。當然實驗室的伙伴們- 永裕學長、佳欣、書綺、志仁、易呈、劉耘、雁婷、慧妍、易澤、黃姐的幫忙，也是我能完成論文的重要助力，謝謝你們。最後要感謝我的家人們，謝謝你們的關心、支持、與包容。謹將此份榮耀與喜悅與你們分享。

摘　要

在黃果蠅(*Drosophila melanogaster*), 馬得拉蜚蠊(*Leucophaea maderae*), 及黃斑黑蟋蟀 (*Gryllus bimaculatus*), 色素散佈因子被時鐘細胞作為輸出訊息的分子，也是使時鐘細胞能同步運行的協調因子。本論文研究是在探討色素散佈因子在德國蜚蠊的功能。首先，進行了色素散佈因子 cDNA 的選殖。色素散佈因子 cDNA 的序列及結構和小翅苯蝗 (*Romalea microptera*) 及寒蟬 (*Meimuna opalifera*) 的色素散佈因子 cDNA 較為相似；與黃果蠅及家蠶 (*Bombyx mori*) 之色素散佈因子 cDNA 的相似度較低。分析蜚蠊腦內色素散佈因子基因的表現型式，發現色素散佈因子基因的表現並不會呈現日週律動。此結果與黃果蠅之色素散佈因子基因的表現形式相似。色素散佈因子之前驅蛋白質是由 87 個胺基酸構成，在序列及結構上仍是與小翅苯蝗及寒蟬的色素散佈因子前驅蛋白質較為相似。選殖出色素散佈因子 cDNA 後，就接著進行核糖核酸干擾實驗，藉由注射色素散佈因子的雙股核糖核酸來降低色素散佈因子基因的表現。從注射雙股核糖核酸的第二天起，色素散佈因子基因的表現就顯著地降低，而且這個抑制作用至少能持續 56 天之久。藉由組織免疫染色法，可見到實驗組蜚蠊之時鐘細胞內色素散佈因子的量明顯降低。由行為方面的研究結果可知，不論是處於有光暗週期或全暗環境的德國蜚蠊雄蟲，在注射色素散佈因子的雙股核糖核酸之後，其活動的日週律動都會消失。但同樣的處理，對原本就無法表現出活動日週律動的雌蟲，則無明顯的影響。進一步分析雄蟲的活動記錄，發現雄蟲的每日活動總量會隨著日齡的增加而增多，但注射色素散佈因子的雙股核糖核酸之後，雄蟲的活動量並未隨著其日齡的增加而明顯增多。此外，夜行性的德國蜚蠊

原本在關燈後一小時內，會有一個明顯的活動高峰出現，但分析注射色素散佈因子的雙股核糖核酸後 11-20 天的雄蟲活動記錄，發現關燈後的活動高峰並沒有出現。若在注射後逐天檢視活動的分佈情形，可見到活動高峰在注射後日漸減低，在七天後就完全消失。由以上的結果推論，在德國蜚蠊，色素散佈因子應是時鐘細胞的訊息輸出因子，用於調控活動行為，使其表現出日週律動。關於注射色素散佈因子的雙股核糖核酸對雌蟲生殖功能的影響，也進行了初步的探討，但結果顯示並無明顯的影響。

關鍵詞: 運動，日週律動，神經胜肽，核糖核酸干擾實驗，基因靜默，德國蜚蠊

Abstract

In *Drosophila melanogaster*, *Leucophaea maderae*, *and Gryllus bimaculatus*, the pigment-dispersing factor (PDF) functions as output and coupling signal of locomotor circadian clocks. In this study, the German cockroach, *Blattella germanica*, was investigated to reveal the functions of PDF. The *pdf* cDNA had been cloned and its sequence and structure showed higher similarity with *Romalea microptera* and *Meimuna opalifera* than that of *D. melanogaster* and *Bombyx mori*. The *pdf* gene in the head of the German cockroach did not express rhythmically which displayed the similar pattern as *D. melanogaster*. The PDF precursor protein of the German cockroach was composed of 87 amino acids and its sequence was also similar to that of *R. microptera* and *M. opalifera*. Once the *pdf* cDNA had been cloned, we explored the functions of PDF in the German cockroach with RNA interference technique. After *pdf* double-stranded RNA (dsRNA) injection, the amount of *pdf* mRNA decreased significantly since the second day and this knockdown effect could persist for at last 56 days. With immunostaining technique, the clock cells of *pdf* dsRNA-injected cockroaches could not be stained by anti-PDF antibody. In the behavioral study, the injection of *pdf* dsRNA caused rhythmic males to become arrhythmic in light-dark cycles or constant darkness, but had no obvious effect on the locomotion of female cockroaches. In addition, due to the nocturnal nature of the German cockroach, the locomotor activity increased after light off or entering subjective night. However, this activity peak gradually disappeared after *pdf* dsRNA injection. Based on these two lines of evidences, PDF serves as an output regulator of locomotor circadian rhythm in the German cockroach. Beside, the

effect of *pdf* dsRNA injection on the reproduction of female cockroaches had also been studied. The results showed that PDF might not be involved in the regulation of reproduction in female cockroaches

Key words: locomotion, circadian rhythm, neuropeptides, RNA interference, gene silencing, *Blattella germanica*

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Chapter 1

Introduction

For most of organisms, the endogenous circadian system enables them to behave rhythmically with the changes of environment everyday. The circadian system is composed of input pathways, circadian clocks, and output pathways. The receptors can transform different kinds of environmental time signals, such as light or temperature, into the electric or chemical signals, and then send these signals into the circadian clock through the input pathways (Zordan et al., 2001). The circadian clock generates internal time signals. When the internal and the external time are different, the circadian clock will be adjusted to synchronize with the time of environment (Roenneberg and Merrow, 2002). The internal time signals are sent to the effectors through the output pathways so that physiological reactions and behaviors can proceed in proper temporal order. The pigment dispersing factor (PDF) has been proposed to be an important output factor of circadian clock in insects (Park, 2000). In this study, the functions of PDF in the German cockroach (*Blattella germanica* L.) were investigated.

1.1 The pigment dispersing factor (PDF)

The PDF peptide was found in the studies on the pigment dispersing hormone (PDH) functions. PDH is a neurohormone of the crustacean. In crustaceans, PDH can trigger the pigment dispersion in the chromatophores of the body integument and the screen pigment migration in the distal pigment cells of the compound eyes (Kleinholz, 1975). In some experiments, they find that the extract of insect heads can evoke the same responses as PDH in the crustacean (Dores and Herman, 1981). Therefore, the effective peptide from the insect is isolated and named PDF (Rao et al., 1987).

Since the sequence of PDF was found to be similar to the β-PDH of the crustacean, the anti-PDH serum had been used to find out the PDH immunoreactive (PDH-ir) cells in many insect species. In 10 species of orthopteroid insects, three groups of PDH-ir neurons have been found in the optic lobes, and one group is located in the anterior edge of the medulla beside the accessory medulla (Homberg et al., 1991). In the *Drosophila melanogaster*, only 16 PDH-ir neurons have been stained, and 8 PDH-ir neurons are located in the optic lobes (Nässel et al., 1993). The locations of the PDH-ir cells were similar to the suggested site of circadian clock. Therefore, many scientists started to investigate the functions of PDF in insects.

1.2 The location of circadian clock

When the left optic lobe of the Madeira cockroach (*Leucophaea maderae*) is removed and the accessory medulla of the right optic lobe is destroyed, the cockroach loses its circadian locomotor rhythm (Sokolove, 1975). After cutting both optic stalks, the Madeira cockroach become arrhythmic (Stengl and Homberg, 1994). However, transplanting an accessory medulla into the antennal lobe can restore the circadian locomotor rhythm of these cockroaches (Reischig and Stengl, 2003). These results indicated that the circadian clock of the Madeira cockroach might be located in the accessory medulla of the optic lobes.

In *D. melanogaster*, many evidences showed that the ventral lateral neurons (LN_vs) which located in the lateral brain were the circadian clock neurons. In the studies of the brain structure mutants, mutants with reduced optic lobes are rhythmic (Helfrich-Förster, 1986), but LN mutants *disconnected* (*disco*) are behaviorally arrhythmic (Dushay et al., 1989). Helfrich-Förster (1998)

finds that few disco mutants with single LN_vs, which its terminals extended into the superior protocerebrum, are rhythmic. While the projection pattern of the LN_vs has been revealed by using antiserum against PDH, their processes widely distribute in the optic lobes and in the superior protocerebrum (Helfrich-Förster, 1995). The *per* mutant strain is locomotor arrhythmic. When the *per* is expressed in the LNs in one of the transgenic lines, their arrhythmic behavior can be rescued (Frisch et al., 1994).

Most of male German cockroaches and ovariectomized females express locomotor circadian rhythm (Lin and Lee, 1996). After both of the optic tracts has been severed, the male German cockroaches lose their locomotor circadian rhythm (Wen and Lee, 2000). Since free-running period of male cockroaches are different under constant light (>24hr; LL) and constant darkness (<24hr; DD), the one optic tract severed males under LL or DD conditions reveal two independent circadian clocks in each optic lobe. Furthermore, these results indicate that two clocks are mutually coupled (Wen and Lee, 2000). In the results of anti-PDF immunostaining, three groups of PDF-ir neurons have been found (Wen and Lee, 2008), and their locations are similar to other species (Homberg et al., 1991). The group that located in the proximal frontoventral region of the accessory medulla shows the strongest immunoreactivity, and is the most possible candidate of the circadian clock (Wen and Lee, 2008).

1.3 The clock genes oscillate in circadian clock

There are several genes express rhythmically in the circadian clock cells. The organism become arrhythmic when these genes mutate (Stanewsky, 2002). These genes are called clock genes, include *cycle*, *Clock*, *timeless*, *period*, etc.

These clock genes form a feedback loop where their cyclic expression generates the internal time signal. Since the circadian locomotor clock cells are neurons, their output signal should be sent via neurotransmitters. The PDF has been proposed to be one of the output molecules.

1.4 The *pdf* gene and cDNA

The *pdf* genes of many insect species have been reported (Park and Hall, 1998; GeneBank: AF110059, FJ043031, FJ154750), but so far the *pdf* gene of *D. melanogaster* is the only one that has been studied functionally assayed. The *pdf* gene of *D. melanogaster* is intronless, and its transcript is predominantly expressed in the head of adult flies. The expression level of the *pdf* transcript does not change over the time course of day and night (Park and Hall, 1998; Park et al., 2000). The expression of *pdf* gene in small LN_vs is regulated by clock genes. In *Clock* and *cycle* mutants, the *pdf* mRNAs in small LN_vs diminish severely and no PDF is detected, while in large LN_vs, neither *pdf* mRNA nor PDF expression level has been influenced. In *period* and *timeless* mutants, the amount of *pdf* mRNA is similar to wild-type flies. These results show that both *Clock* and *cycle* genes appear to be the positive regulators of *pdf* gene in the small LN_vs (Park et al., 2000).

Up to now, the *pdf* cDNA of 7 insect species has been reported (Park and Hall, 1998; Sato et al., 2002; Chuman et al., 2002; Matsushima et al., 2004; GeneBank: AB127943, AB298933, U42472). The size of the deduced PDF precursors ranged from 43 to 105 amino acids, and the PDF precursors could be divided into signal, PDF-associated peptide, and PDF regions except the cricket *Gryllus bimaculatus*. Although the size of these PDF precursors was diverse, the

size of PDF peptides was fixed at 18, and exhibited a high level of sequence identical. In *Drosophila*, the anti-PDH immunoreactivity level in the terminals of small LN_vs oscillates rhythmically. In the LD condition, the peak and trough time are 1 hour after light-on and 1 hour after light-off, respectively, while the anti-PDH immunoreactivity level in the cell body of small LN_vs do not oscillate. In *period* and *timeless* mutants, the cycling of anti-PDH immunoreactivity level in the small LN_vs nerve terminals is abolished. These results suggest that PERIOD and TIMELESS might involve in the post-translational regulation of *pdf* gene in the axonal terminals (Park et al., 2000).

1.5 The potential functions of PDF in insects

PDF is the output signal of locomotor circadian clock. In *Drosophila*, the locomotion of wild-type flies is rhythmic under LD and DD conditions, and shows two peaks, morning and evening peak, in the daily locomotor activity profile. However, *pdf* mutant flies are rhythmic only in the LD condition, and their morning peak of the locomotor activity profile is greatly reduced (Renn et al., 1999). In addition, the behavioral phenotype of PDF receptor mutant is similar to the *pdf* mutant which can be rescued after expressing PDF receptor gene in all clock neurons (Hyun et al., 2005; Lear et al., 2005). When the *pdf* gene is ectopically expressed in the neurons of the dorsal central brain of wild-type flies, some of these flies become arrhythmic or show complex rhythm, whereas ectopically expresses the *pdf* gene only in the LN_vs will not disrupt the circadian locomotor rhythm (Helfrich-Förster et al., 2000). Furthermore, the output projections of LN_vs terminate in the dorsal protocerebrum, and the anti-PDH immunoreactivity level oscillates rhythmically in these terminals

(Park, et al., 2000). The results of in vitro PDF peptide binding assay show that PDF can bind to the dorsal neurons 3 (DN_3) within the superior protocerebrum region (Peng et al., 2003). The immunostaining results on anti-PDF receptor reveal that the PDF receptor is localized at DN_1 and DN_3 (Hyun et al., 2005; Mertens et al., 2005). Based on these results, the PDF serves as an output signal to regulate the locomotor circadian clock in the brain.

PDF is involved in the coordination of circadian clocks. After injecting synthetic PDH into the vicinity of the accessory medulla (AMe), the phase of the circadian locomotor rhythm will be reset in *L. maderae* (Petri and Stengl, 1997). Since the AMe is the location of cell body of clock neurons, this result indicates that PDF might serve as the coupling signal of the locomotor circadian clock in *L. maderae.* In the extracellular recording, action potentials with regular interspike interval have been recorded. The cells within the AMe form different assemblies. Cells share the same phase within an assembly, whereas cells among assemblies are different in phase. Application of PDF on the in vitro AMe can lock the phase and synchronize different cell assemblies (Schneider and Stengl, 2005). The *pdf* mutant of *Drosophila* can not maintain locomotor circadian rhythm under DD condition. By using time series immunostaining, Lin *et al.* (2004) find that the phase of PERIOD expression among the small LN_vs neurons is disrupted in *pdf* mutant flies. These results reveal that PDF is also involved in the coordination between cells within a circadian clock.

The medulla bilateral neurons (MBNs) of cricket *G. bimaculatus* are the interneurons to connect the bilateral medulla, which have been suggested to be the interactive pathway of two circadian clocks. The photo-responsiveness of the MBNs shows a distinctive day/night changes, which shows response greater

during the night. The cell bodies of MBNs are located in the optic lobes. After injecting PDF into the optic lobe, the photo-responsiveness of the MBNs increases significantly during the day, whereas injecting at night only has little influence. Injection of anti-PDF IgG reduces the photo-responsiveness of MBNs in both day and night. According to these results, Saifullah and Tomioka (2003) suggest that PDF is released during the night and set the night state of the coupling pathway.

PDF is necessary for sustained molecular clock oscillation in constant darkness. The *pdf* mutant of *Drosophila* expresses locomotor circadian rhythm under LD condition, but is arrhythmic under DD condition. In LD, the *timeless* and *cryptochrome* genes oscillate rhythmically in *pdf* mutant flies. However, the molecular oscillation of both genes sustains in the first three days of DD, but the oscillation is gradually lost after the fourth day in DD in all clock neurons (Peng et al., 2003). This result indicates the essential role of PDF in molecular clock oscillation under DD condition.

Only in *G. bimaculatus*, there is a nuclear localization signal in the N-terminus of the PDF precursor (Chuman, et al., 2002). The anti-PDF immunoreactive signal exhibits not only cytoplasmic but also nuclear. After COS-7 cells are transfected by constructed plasmid, which can encode the green fluorescent protein (GFP) that is fused to the C-terminus of PAP-PDF, the PAP-PDF-GFP can translocate into the nucleus. When the nuclear localization signal is mutated, there will be no distinct nuclear translocation of the fused GFP. These results suggest that PDF might have functions within the nucleus, but its role is still unknown.

1.6 The aim of this study

The majority of research works on the circadian rhythm of the insect are done in the model species of *Drosophila*. Based on the great diversity of insect species, the mechanisms underlying circadian rhythm among different species should display great diversity too. Since *Drosophila* is the evolutionary advanced group of species, its circadian mechanisms may not represent the typical model for insect species. Therefore, investigating the mechanism of circadian rhythm in insects other than *Drosophila* becomes necessary. In addition, many studies have been done in the living fossil species of cockroach, the German cockroach becomes advantage as the object of the study.

The object of this research is to reveal whether the PDF peptide involving in the circadian system of the German cockroach or not. Firstly, the *pdf* gene must be cloned, in order to know the characters of the *pdf* gene and PDF peptide. Secondly, various experiments could be designed to unveil the functions of PDF in the circadian system according to the knowledge of *pdf* gene and PDF peptide.

Chapter 2

Cloning and Analyzing the *pdf* Gene of the German Cockroach, *Blattella germanica* (L.)

2.1 Introduction

Pigment dispersing factor (PDF) is a neuropeptide which is first identified from head extraction of an insect (Rao et al., 1987). It is composed of 18 amino acids, and its sequence is similar to the β-pigment dispersing hormone (PDH) of crustacean (Rao et al., 1991; Rao and Riehm, 1993). In crustaceans, PDH can trigger the pigment dispersion in the chromatophores of the body integument and the screening pigment migration in the distal pigment cells of the compound eyes (Kleinholz, 1975). When PDF is injected, it can induce the same response in crustaceans (Rao et al., 1991).

The immunostaining result showed that only limited neurons in the brain and optic lobe express PDF (Homberg et al., 1991; Nässel et al., 1993; Petri et al., 1995), and the location of the PDF-expressed neurons is similar to the suggested location of circadian clock (Sokolove, 1975; Stengl and Homberg, 1994). These results strongly reveal that PDF should be involved in the mechanism of expressing circadian rhythm.

The *pdf* cDNA and gene of *Drosophila melanogaster* is the first one to be cloned (Park and Hall, 1998). Up to now, *pdf* cDNAs of four more insect species have been cloned and reported (Chuman et al., 2002; Sato et al., 2002; Matsushima et al., 2004; GeneBank: U42472). The *pdf* gene of *Drosophila* is intronless, and the *pdf* transcript is predominantly expressed in the head of the adult flies. The expression level of the *pdf* transcript does not change over the time course of day and night (Park and Hall, 1998; Park et al., 2000). The expression of *pdf* gene in small LN_vs is regulated by clock genes. In *Clock* and *cycle* mutants, the *pdf* mRNA in small LN_vs diminishes severely and no PDF can be detected, while in large LN_vs, neither *pdf* mRNA nor PDF expression level

has been influenced. In *period* and *timeless* mutants, the amount of *pdf* mRNA is similar to wild-type flies. These results reveal that both *Clock* and *cycle* genes appear to be the positive regulators of *pdf* gene in the small LN_vs (Park et al., 2000).

The sizes of the five deduced PDF precursors range between 43 and 102 amino acids (Park and Hall, 1998; Chuman et al., 2002; Sato et al., 2002; Matsushima et al., 2004; GeneBank: U42472). The structure of PDF precursor could be divided into signal, PDF-associated peptide (PAP), and PDF regions except in the cricket *Gryllus bimaculatus*. Although these PDF precursors were different in length, but the PDF peptides had fixed number of 18 amino acids and exhibited a high degree of identical sequence. In *Drosophila*, the anti-PDH immunoreactive level in the terminals of small LN_vs oscillates rhythmically. In the LD condition, the peak time is 1 hour after light-on, and the trough time is 1 hour after light-off. However, the anti-PDH immunoreactive level in the cell body of small LN_vs does not oscillate. In *period* and *timeless* mutants, the cycling of anti-PDH immunoreactive level in the small LN_vs nerve terminals is abolished. These results suggest that PERIOD and TIMELESS might be involved in the posttranslational regulation of *pdf* gene in the axonal terminals but not in cell body (Park, et al., 2000).

The previous reports reveal that the German cockroach could express circadian in many aspects (Lin and Lee, 1996; Chang and Lee, 2001; Lin et al., 2002). In order to know whether PDF is involved in the circadian system of the German cockroach in control of locomotion, cloning the *pdf* cDNA is the first logic step to be taken.

2.2 Materials and methods

2.2.1 Insect

German cockroaches (*Blattella germanica* L.) were reared in an environmental chamber at 28ºC with a 12:12 light/dark (LD 12:12) schedule. Water and food were provided *ad libitum*. To determine the age of experimental cockroaches, adults for the following experiments were collected within 24 hour of emergence and kept in a transparent plastic jar (14 x 14 x 20 cm).

2.2.2 RNA extraction

After removing antennae, the head of a cockroach was severed and put into 100 µl TRIZOL® reagent (Invitrogen). After homogenizing, sample was placed at room temperature for 5 minutes, and then added 20 µl chloroform and shook the mixture vigorously by hand for 15 seconds. Placed the mixture at room temperature for 5 minutes, then centrifuged at 12000 g for 15 minutes at 4ºC. Following centrifugation, the upper aqueous phase was transferred into a microtube. An equal volume of isopropanol was added and inverted to mix. Placed the mixture at room temperature for 10 minutes, then centrifuged at 12000g for 10 minutes at 4ºC. Removed the supernatant and added 90 µl of 75% ethanol to wash the pellet, and then centrifuged at 7500 g for 5 minutes at 4ºC. Discarded the supernatant and air-dry the RNA pellet for 5-10 minutes. The RNA pellet was dissolved in 10 µl diethyl pyrocarbonate treated water (DEPC H_2O).

2.2.3 Reverse transcription (RT)

The mRNA was reverse-transcripted into cDNA by Superscript III reverse transcriptase (Invitrogen). For 3' RACE, added 8 µl RNA , 1 µl 50 µM Ad-dT17 primer

(5’-GGCCACGCGTCGACTAGTAC-T_{17}-3’), and 1 µl 10 mM dNTP Mix into a 0.2 ml microtube. Kept the mixture at 65ºC for 5 minutes and then placed it on ice for more than 1 minute. After brief centrifugation, added 2 µl 10X First-Strand Buffer, 4 µl 25 mM $MgCl_2$, 2 µl 0.1 M DTT, 1 µl RNaseOUT™ Recombinant RNase Inhibitor (40 units/µl) and 1 µl of SuperScript™ III reverse transcriptase (200 units/µl). Mixed gently, and then incubated the mixture at 50ºC for 60 minutes, followed by 85ºC for 15 minutes. For 5’ region cloning, primer GS3-2 (5’-CATTCCCCACTCATACATAC-3’) has been used to replace the Ad-dT_{17} primer. GS3-2 was a gene specific primer, its sequence was complementary to 3’ untranslated region of *pdf* cDNA.

2.2.4 3’ RACE

The primer PDF5-3 (5'-AAGCGCAACTC(G/T)GA(A/G)(A/C)T(A/C)ATCAA(T/C)TC -3') for 3’ RACE was designed according to known sequences of some insect species. The sequence of PDF5-3 was picked up from open reading frame which was close to PDF peptide coding region. The PCR mixture (25 µl) for 3’ RACE included 1 µl of reverse transcription product, 10 pmol of Ad primer (5’-GGCCACGCGTCGACTAGTAC-3’), 10 pmol of degenerate primer, and 5 µl 5X Taq Master Mix (Protech). PCR (25 cycles) was performed as follows: denaturation at 94ºC for 1 minute, annealing at 60ºC for 1 minute, extension at 72ºC for 1 minute. The PCR products were identified on a 1.5% agarose gel. The candidate DNA molecules were purified from the gel by using DNA extract kit (Viogene). The purified DNA molecules were cloned into the pGEMTeasy vector (Promega). DNA sequencing was done by Tri-I Biotech.

2.2.5 5' region cloning

The 5' region of *pdf* cDNA was cloned by using terminal transferase. The total RNA of the head of the German cockroach was reverse transcribed with primer GS3-2. The product of reverse transcription was cleaned up by using GFX PCR DNA and Gel Band Purification Kit (Amersham, GE). After the clean-up process, the poly-dT tail was added to the 3' end of cDNA by using terminal transferase. For tailing reaction, 38.5 μl clean-up cDNA, 5 μl 10 X NEBuffer 4, 5 μl 2.5 mM $CoCl_2$, 1 μl 10mM dATP (GeneMark), and 0.5 μl terminal transferase (NEW ENGLAND BioLabs) were mixed. The mixture was placed at 37ºC for 15 minutes and then inactivated the terminal transferase by incubating the mixture at 70ºC for 10 minutes.

The PCR mixture (25 μl) for 5' region cloning included 1 μl of reverse transcription product, 10 pmol of Ad-dT_{17} primer, 10 pmol of GS3-2 primer, and 5 μl 5X Taq Master Mix (Protech). PCR (25 cycles) was performed as follows: denaturation at 94ºC for 1 minute, annealing at 55ºC for 1 minute, extension at 72ºC for 3 minutes. The PCR products were identified on a 1.5% agarose gel. The candidate DNA molecules were purified from the gel and cloned into the pGEMTeasy vector (Promega). DNA sequencing was done by Tri-I Biotech.

2.2.6 Genomic DNA extraction

The genomic DNA was extracted by using DNA extraction kit (Stratagene). The head and thorax portion of a cockroach was put into 300 μl solution 2 and cut into small pieces by a pair of sterile scissors. Added 0.2 μl pronase and incubated at 55 ºC for 2 hours. Put the sample on ice for 10 minutes and then added 170 μl solution 3. Inverted the sample several times and incubated on ice for 5 minutes. The sample was

centrifuged at 2000g for 15 minutes at 4ºC. The supernatant was transferred to a sterile microtube and 0.8 μl RNase was added to the supernatant. Incubated the sample at 37ºC for 15 minutes. Added 300 μl ice-cold isopropanol and inverted the mixture several times gently. The mixture was centrifuged at full speed for 3 minutes. Discarded the supernatant and washed the pellet with 300 μl 70% ethanol. The sample was centrifuged at full speed for 1 minute. Discarded the supernatant and air-dry the pellet. Adding 40 μl sterile H_2O to dissolved the pellet. The quality of the genomic DNA was checked by electrophoresis on 0.8% agarose gel and the quantity was estimated by the value of OD_{260}.

2.2.7 Inverse PCR

The upstream and intron sequences were determined by inverse PCR. The genomic DNA was cut by the restriction enzyme (RE). Sixteen kinds of REs have been selected, including BfaI, ClaI, EcoRI, Hha, Hin61, Hind III, HpaII, KpnI, MboI, MluI, MseI, MunI, NdeI, NlaIII, TaiI, and Taqα I (NEW ENGLAND BioLabs). The sizes of recognition sequence of these REs were between 4 to 6 bp, and a protruding end would form after RE digestion. The mixture (50 μl) for restriction enzyme digestion including 5 μl 10X NEBuffer, 0.5 or 1 μl restriction enzyme, 4 μg genomic DNA. The mixture was incubated at the suggested temperature for overnight. After digestion, the reactions were stopped by incubating at 80ºC for 20 min or phenol/chloroform treatment.

Next, the RE-digested genomic DNA fragments were self-ligated into the ring form. The mixture (25 μl) for self-ligation reaction contained 2.5 μl 10X buffer, 5 μl RE-digested genomic DNA, and 0.5 μl ligase. The mixture was incubated at 16ºC for overnight. The reaction was stopped by incubating the mixture at 65ºC for 10 minutes.

The primers for inverse PCR were designed according to the *pdf* cDNA (Fig. 2-2). The PCR mixture (25 μl) included 1 μl self-ligated genomic DNA fragments, 10 pmol of forward primer, 10 pmol of reverse primer, and 5 μl 5X Taq Master Mix (Protech). The annealing temperature was set based on the sequence of the primer pair. The PCR products were identified on a 1.5% agarose gel. The candidate DNA molecules were purified from the gel by using DNA extract kit (Viogene). The purified DNA molecules were sequenced by Tri-I Biotech.

2.2.8 RT-PCR for checking *pdf* gene expression

The heads of male cockroaches were severed at various time point within one day. The total RNA of each head was extracted with TRIZOL® reagent (Invitrogen). The RNA pellet was dissolved in 10 μl DEPC H_2O. Eight microliter RNA was reverse transcribed by using Superscript III reverse transcriptase (Invitrogen) and the oligo$(dT)_{20}$ primer. The PCR mixture (25 μl) for amplifying *pdf* fragment included 1 μl of reverse transcription product, 10 pmol of forward primer (5'-CTGCCATTCAACTGGAAGACGA-3'), 10 pmol of reverse primer (5'-AGCATTCCCCACTCATACATACAAAATC-3'), and 5 μl 5X Taq Master Mix (Protech). PCR (25 cycles) was performed as following: denaturation at 94ºC for 1 min, annealing at 60ºC for 1 min, extension at 72ºC for 1 min. Beside, PCR for amplifying *actin* fragment had also been performed. The PCR mixture (25 μl) included 1 μl of 10 times diluted reverse transcription product, 10 pmol of forward primer, 10 pmol of reverse primer, and 5 μl PCR mixture. The PCR program was described as above. The PCR products were identified on a 1.5% agarose gel.

2.2.9 Data analysis

The analysis of the *pdf* gene, the sequence alignments and similarity calculation of cDNAs and PDF precursors were processed by the software Vector NTI (suite 8, Invitrogen). The structure and characters of the PDF precursors were predicted by program SignalP 3.0 (Bendtsen et al., 2004; http://www.cbs.dtu.dk/services/SignalP/) and PSORT II (http://psort.ims.u-tokyo.ac.jp/form2.html).

2.3 Results

2.3.1 The characterization of the *pdf* cDNA of the German cockroach

Two types of *pdf* cDNA have been cloned from the heads of the German cockroach, one was 474 base pair (bp) long and the other was 478 bp long (Fig. 2-1). Except for the extra four bases (base 61-64) in the clone II, the remaining sequence of the cDNA was identical to the clone I. The *pdf* cDNA (clone I) sequences of the German cockroach and other 7 insect species have been compared (Table 2-1). Except for the *D. melanogaster* and *Phormia regina*, the identities of entire *pdf* cDNA among German cockroach and other 5 species were akin to 50% (50%-56%). When the comparing region was limited to the open reading frame, the sequence of the German cockroach was similar to *Meimuna opalifera* and *Romalea microptera* which showed 65% and 61%, respectively, in similarity (Table 2-2). The similarities among the German cockroach and the remaining 5 species were akin to 40% (36%-44%) (Table 2-2).

2.3.2 Structure of the *pdf* gene

The *pdf* gene contained 4 exons and 3 introns (Fig. 2-2). The sizes of the four exons were 4, 56 or 60, 71 and 143 bp, respectively. The open reading frame of *pdf* cDNA was located in the third exon. According to the analysis using software Vector NTI, the TATA box (TATAAA) was located in the upstream of *pdf* gene (-16 to -21 bp), but no transcription factor binding motifs could be found within the 348 bp upstream region. The polyadenylation signal (AATAAA) was located in the 3' untranslated region (455 to 460 bp) (Fig. 2-2).

2.3.3 PDF precursor protein and PDF peptide

The predicted sequence of the PDF precursor protein in the German cockroach was composed of 87 amino acids (Fig. 2-1). According to the analysis of SignalP 3.0, the structure of the PDF precursor protein could be divided into three parts (Fig. 2-3): the signal peptide (1-19 amino acids, SP), the PDF associated peptide (20-64 amino acids, PAP) and PDF peptide (67-84 amino acids). The 65th and 66th amino acids (KR) were the cleavage site for producing PDF peptide. The last 3 amino acids (GRK) of the C-terminal represented the amidation site. Except for the *G. bimaculatus,* the structures of the PDF precursor proteins of another 8 species were similar which all composed of SP, PAP, and PDF. The similarity of the primary structure of PDF protein precursor among 9 insect species was shown in Table 2-3 and Figure 2-4. For the whole precursor sequence, the German cockroach was more similar to the *R. microptera* (55% similarity), and *M. opalifera* (50% similarity). As for the sequence of PDF, the similarities were very high among 11 species. (Table 2-4, Fig. 2-5). The possible location of PDF precursor protein has been predicted by the PSORT II program. The precursor protein of the German cockroach was extracellular (66.7%, Table 2-5). This result indicated that PDF of the German cockroach was a secretory peptide.

2.3.4 The *pdf* gene expression pattern

For determining the expression pattern of *pdf* gene in the German cockroach, the total RNA of the head was extracted at different time point under LD cycles (Fig. 2-6). The amount of *pdf* cDNA was similar at different time points within a day. The expression of *pdf* gene did not show circadian rhythm at the transcription level.

2.4 Discussion

The *pdf* gene of *B. germanica* contained 3 introns, the first and second introns located in the 5' untranslated region, and the third introns located in 3' untranslated region. The *pdf* genes of the *Musca domestica* (GeneBank: FJ043031) *and Anopheles gambiae* (GeneBank: FJ154750) also contained at least one intron, while the *pdf* gene of *D. melanogaster* contained no introns (Park and Hall, 1998). Although we still do not know whether the introns would have any influences on the expression of *pdf* gene or not, this difference revealed that *D. melanogaster* was unique in this aspect.

Two clones of *pdf* cDNA in the German cockroach had been found in this study. The structures of these two cDNAs were identical except the only difference in the 5' untranslated region, where the clone II was 4 bases longer than the clone I (Fig. 2-1). The sequence of this difference was "GTAG", and the start sequence of an intron was also "GT". Beside, the intron 2 was next to this 4-bases difference (Fig. 2-2). Therefore, during the intron 2 splicing, the possibilities of cutting at the first and the second "GT" sites were similar. When the spliceosome cut the intron 2 at the first "GT" site, the cDNA clone I would be produced; the cDNA clone II formed as the spliceosome cut at the second "GT" site.

In previous reports, the PDF precursors have been classified into three groups: the D-M group, the M-R group, and the G group (Matsushima et al., 2004). The D-M group contained the PDF precursors of *D. melanogaster* and *M. domestica*. The sizes of the PDF precursors of *D. melanogaster* and *M. domestica* were 102 and 97 amino acids, respectively, which were the longest of the three groups. The M-R group contained the PDF precursors of *R. microptera and M. opalifera.* The sizes of the PDF precursors of the *R. microptera and M. opalifera* were 89 and 82 amino acids, respectively. The G group contained the PDF precursors of *G. bimaculatus*. The sizes of the PDF precursor

of the *G. bimaculatus* was the shortest, contained only 43 amino acids. The sequence identity was both high in D-M group and M-R group (Fig. 1-4). The PDF precursor of *B. germanica* was composed of 87 amino acids. This peptide would be classified into the M-R group according to its size. According to the calculation of program Vector NT1, the identity of the PDF precursors of M-R group was 47.2% before the addition of *B. germanica*. Although the sequence identity decreased to 35.1% after the adding *B. germanica*, it still showed high sequence identity. Therefore, it was proper to classify *B. germanica*, *M. opalifera*, and *R. microptera* as a group. Up to now the PDF precursors of *Bombyx mori* (GeneBank: AB298933), *P. regina* (GeneBank: AB127943), *A. gambiae* (GeneBank: FJ154750) have also been reported. According to the sizes of the PDF precursors of *B. mori* (n = 103), *P. regina* (n = 92), and *A. gambiae* (n = 105), these three peptides would be classified into the D-M group. At first, the sequence identity of D-M group was 36.9%. The adding *P. regina* did not change the sequence identity (from 36.9% to 35.9%). Therefore, it was proper to classify *P. regina* into the M-D group. However, the addition of *B. mori and A. gambiae* would reduce the sequence identity to 17.5% and 22.2%, respectively. So putting them into the D-M group might be not so proper. Putting *B. mori and A. gambiae* into the M-R group or G group, all the sequence identities would not exceed 20%. So the PDF precursors of *B. mori and A. gambiae* could not be included in any one of these three groups. In other words, as there were more PDF precursor sequences being reported, the three group system would not be enough for classifying all the PDF precursors.

Based on the prediction by software PSOT II, the subcellular localization sites of the PDF precursor proteins were extracellular, except in the *G. bimaculatus*. The results of anti-PDH and anti-PDF immunostaining indicated that immunoreactivity can be detected both in the cytoplasm of cell body and neurites, but not in the nucleus

(Homberg et al., 1991; Nässel et al., 1993; Petri et al., 1995; Sehadova et al., 2003; Wen and Lee, 2008). In *G. bimaculatus*, the PDF precursor is quite short and bears no PAP. The sequence of the SP of the PDF precursor reveals to be the nuclear localization signal, and this property has been proved by using PDF-GEP fusion protein and cell culture (Chuman et al., 2002). While in results of the anti-PDF immunostaining of the brain of *G. bimaculatus*, the immunoreactivity is still cytoplasmic, but not nuclear (Abdelsalam et al., 2008).

The PDF peptide of the German cockroach was also composed of 18 amino acids. The 1st - 7th, 9th, 10th , 12th and 18th amino acids are identical among all known β-PDHs of the crustacean (Matsushima et al., 2004). In the German cockroach, the 1st - 3rd, 5th - 7th, 9th, 10th , 12th and 18th amino acids of the PDF peptide were identical to those of β-PDH of the crustacean, but the 4th amino acid was different (Ile ↔ Leu). The substitution at position 4 (Ile ↔ Leu) could also be found in the PDF peptide of *Acheta domesticus*, *M. opalifera and R. microptera* (Fig. 2-5).

When aligned all the PDF precursors, we could find that the PDF regions showed very high identity among all the precursors (Fig. 2-4). The sequence identity of the 11 known PDF peptides was 66.7%. The sequence of the PDF of *B. mori* was more different from other PDFs. If excluded the PDF of *B. mori* from the group, the sequence identity would rise to 77.8% with only 4 sites differences. When these 10 PDFs were further grouped by the similarity, the result was basically consistent with the taxonomic relationships among these insects (Fig. 2-5). According to the 10th amino acid, the PDFs could be divided into two groups. The 10th amino acid of the first group was Gly, which was preserved among all known PDHs. The first group included *B. germanica*, *Periplaneta americana*, *R. microptera*, *M. opalifera*, *G. bimaculatus*, and *A. domesticus*. Within this group, the PDF of *B. germanica* was very similar to the PDF of *P.*

Americana. The only difference was at the 15th amino acid (Ile ↔ Leu). From phylogenetic view, *R. microptera* (Order Orthoptera) was closer to *M. opalifera* and *A. domesticus* (Order Orthoptera) than *M. opalifera* (Order Hemiptera), but the sequence of the PDF of *M. opalifera* was identical to that of *M. opalifera* and *A. domesticus*, and the sequence of the PDF of *R. microptera* was different at 14th amino acid (Val ↔ Leu). In the second group, including *D. melanogaster*, *M. domestica*, *P. regina,* and *A gambiae* (Order Dipterathe), 10th amino acid was Ser. Among these four PDFs, the 14th amino acid was the only site of difference. The 14th amino acid was Asn, Ser, or Thr, which were all hydrophilic. The PDF of *B. mori* was the most diverse one. The second and third amino acids were conserved in all known insect PDF and crustacean β-PDH (Matsushima et al., 2004), but *B. mori* was the only exception. Since the PDF of *B. mori* was the only reported case of the Order Lepidoptera, so more data were needed to judge this difference was universal in the Order Lepidoptera or a special case in Hexapoda.

In the transcription level, the expression of the *pdf* gene of the German cockroach did not show rhythmic change within one day (Fig. 2-6). This result was consistent with the *pdf* gene expression pattern of D. *melanogaster*. In the transcription level, the amount of *pdf* mRNA does not show rhythmic fluctuation over the time course of day (Park and Hall, 1998). In the translation level, the immunostaining intensity fluctuates rhythmically in the nerve terminals of *s*-LNvs, the peak time is 1 hour after light on and the trough time is 1 hour after light off. While the immunostaining intensity does not show rhythmic change in the cell bodies of *s*-LNvs. These results suggests that the *s*-LNvs of D. *melanogaster* secrete the PDF rhythmically (Park et al., 2000). But the situation in *Leucophaea maderae* is different, no rhythmic change of the amount of PDF can be monitored in any regions of the brain and optic lobes (Hamasaka et al., 2005). In

G. bimaculatus, the total content of PDF in the optic lobes and cerebral lobes show circadian rhythm. The amount of PDF is higher during the dark phase and lower during the light phase (Abdelsalam et al., 2008). The PDF expression patterns were highly diverse among insect species. This suggests that different regulatory mechanisms have been evolved and PDF plays different roles among insect species.

Table 2-1. The sequence identity of the *pdf* cDNAs among 8 insect species.

	Sequence identity (%)							
Species†	Bg	Rm	Bm	Dm	Pr	Md	Gb	Mo
Bg	100	54	51	27	42	50	56	49
Rm		100	40	26	40	41	49	46
Bm			100	20	36	43	48	42
Dm				100	34	34	23	31
Pr					100	59	39	44
Md						100	49	48
Gb							100	46
Mo								100

† Abbreviations used: Bg, *Blattella germanica*; Bm, *Bombyx mori*; Dm, *Drosophila melanogaster*; Gb, *Gryllus bimaculatus;* Md, *Musca domestica*; Mo, *Meimuna opalifera*; Pr, *Phormia regina;* Rm, *Romalea microptera*.

Table 2-2. The sequence identity of the open reading frame of the *pdf* cDNAs among 8 insect species.

	Sequence identity (%)							
Species†	Bg	Rm	Bm	Dm	Pr	Md	Gb	Mo
Bg	100	61	36	38	44	43	43	65
Rm		100	38	45	46	41	40	61
Bm			100	38	37	36	22	35
Dm				100	48	48	25	39
Pr					100	69	34	44
Md						100	28	41
Gb							100	46
Mo								100

† The abbreviations of species were described in Table 2-1.

Table 2-3. The sequence identity of the PDF precursors among 9 insect species.

	Sequence identity (%)								
Species†	Bg	Bm	Dm	Md	Pr	Ag	Gb	Mo	Rm
Bg	100	29	34	38	40	32	45	50	55
Bm		100	29	23	25	22	23	31	29
Dm			100	42	50	33	26	30	30
Md				100	68	35	29	34	30
Pr					100	35	34	33	34
Ag						100	25	27	24
Gb							100	48	45
Mo								100	58
Rm									100

† Abbreviations used: Ag, *Anopheles gambiae*; the rest abbreviations of species were described in Table 2-1.

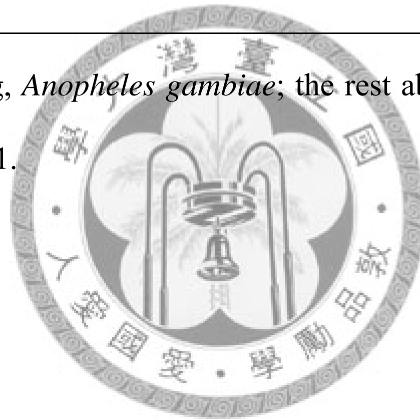

Table 2-4. The sequence identity of the PDF peptides among 11 insect species.

	Sequence identity (%)										
Species†	Bg	Bm	Dm	Md	Pr	Ag	Gb	Mo	Rm	Ad	Pa
Bg	100	72	83	83	83	83	89	89	83	89	94
Bm		100	78	78	78	78	67	67	67	67	72
Dm			100	94	100	94	78	78	78	78	83
Md				100	94	94	78	78	78	78	83
Pr					100	94	78	78	78	78	83
Ag						100	78	78	78	78	83
Gb							100	100	94	100	94
Mo								100	94	100	94
Rm									100	94	89
Ad										100	94
Pa											100

† Abbreviations used: Ad, *Acheta domesticus*; Pa, *Periplaneta americana*; the rest abbreviations of species were described in Table 2-1.

Table 2-5. The predicted subcellular localization sites of the PDF precursor proteins in different species by software PSOT II.

	Subcellular location sites				
	Extracellular	Nuclear	Cytoplasmic	Endomembrane system[a]	Mitochondrial
B. germanica	66.7 %	0 %	11.1 %	22.2 %	0 %
R. microptera	55.6 %	0 %	11.1 %	33.3 %	0 %
M. opalifera	55.6 %	11.1 %	11.1 %	11.1 %	11.1 %
G. bimaculatus	0 %	43.5 %	8.7 %	4.3 %	43.5 %
D. melanogaster	55.6 %	11.1 %	0 %	33.3 %	0 %
P. regina	55.6 %	22.2 %	11.1 %	0 %	11.1 %
M. domestica	44.4 %	22.2 %	11.1 %	0 %	22.2 %
B. mori	33.3 %	33.3 %	22.2 %	0 %	11.1 %

[a] Endomembrane system includes the endoplasmic reticulum, Golgi body, and vacuoles.

```
Clone I   GAAAGTAGTCAGAGAATTTCCTTCTCGCCATATAGTCTTCTCTTG  45
Clone II  GAAAGTAGTCAGAGAATTTCCTTCTCGCCATATAGTCTTCTCTTG  45

Clone I   TCTTCAAGTCAACTA----CAATGAAACACTTGGGAACCATCATC  86
Clone II  TCTTCAAGTCAACTAGTAGCAATGAAACACTTGGGAACCATCATC  90
                              M  K  H  L  G  T  I  I

Clone I   CTGTTTCTATATCTCCTAAGAATGGCTTTCACGTCTCCTGCCATT 131
Clone II  CTGTTTCTATATCTCCTAAGAATGGCTTTCACGTCTCCTGCCATT 135
           L  F  L  Y  L  L  R  M  A  F  T  S  P  A  I

Clone I   CAACTGGAAGACGACAGATATATGGATAAGGAGTTTCAGACGAAT 176
Clone II  CAACTGGAAGACGACAGATATATGGATAAGGAGTTTCAGACGAAT 180
           Q  L  E  D  D  R  Y  M  D  K  E  F  Q  T  N

Clone I   GCTGTAAATGCACGAGAGCTTACCAACTGGATAATGCAGATATTA 221
Clone II  GCTGTAAATGCACGAGAGCTTACCAACTGGATAATGCAGATATTA 225
           A  V  N  A  R  E  L  T  N  W  I  M  Q  I  L

Clone I   ATGCACAAGGGTGAGCCCACAGTCTGCACCCATAAGCGCAACTCA 266
Clone II  ATGCACAAGGGTGAGCCCACAGTCTGCACCCATAAGCGCAACTCA 270
           M  H  K  G  E  P  T  V  C  T  H  K  R [N  S]

Clone I   GAACTCATCAATTCCCTTCTGGGGCTTCCCAAAGTAATCAATGAT 311
Clone II  GAACTCATCAATTCCCTTCTGGGGCTTCCCAAAGTAATCAATGAT 315
          [E  L  I  N  S  L  L  G  L  P  K  V  I  N  D]

Clone I   GCAGGGAGAAAATAAATCAAATTGTAATACAACAACCAGAAACTC 356
Clone II  GCAGGGAGAAAATAAATCAAATTGTAATACAACAACCAGAAACTC 360
          [A] G  R  K

Clone I   CCCTTTTCATCTGTACAAAAAATGTTTTCTTACCACTTTTGTTTA  401
Clone II  CCCTTTTCATCTGTACAAAAAATGTTTTCTTACCACTTTTGTTTA  405

Clone I   TAATATTTTATCATGATTTTGTATGTATGAGTGGGGAATGCTAAA  446
Clone II  TAATATTTTATCATGATTTTGTATGTATGAGTGGGGAATGCTAAA  450

Clone I   TCTCAATAAAGAAACAAAATAAACTCAT  474
              ------
Clone II  TCTCAATAAAGAAACAAAATAAACTCAT  478
              ------
```

Figure 2-1. The nucleotide and deduced amino acid sequence of the *pdf* cDNA of the German cockroach. The potential polyadenylation signal is underlined. The capital letters in the black box indicate the PDF peptide.

tattacaggtcatgtaactaaacaaatgcagttctgagatagaaattagaactca 55

agacaggactgggaatcaaatccagcccatggaatagaaccatatgcactaccaa 110

ctaaaaagaaataagttgtaagtatttaccaattaacaatgactaacatgtgttt 165

taaatcacactgagttttccacactttgtttacatatactctatatgaaatgtca 220

tgtgaggtgcaaggatatctattgtttgatatcttttatacatcttgcgagagca 275

GL-3

aaaataaacagtaaaataataatgcactcaaatagaatagaggtatgtgttatat 330

aaacccttgttgagtgaaGAAAgtgaattgatttctgtccagaacaagacaaca 385

agagggggtggcaatggacctcctgctgcttcgttaccggcccactctctcaaac 440

GR-2

tcctgcatttgatggcatcaacgcatcacaacctcttataataatccaagtagct 495

tcttttaaaatatcaatttcctcccccaaggggagagggtatcggaatttccag 550

GS5T

tacttaaggcagccacattctcaccaattctGTAGTCAGAGAATTTCCTTCTCGC 605

G5-1 IL-2

CATATAGTCTTCTCTTGTCTTCAAGTCAACTAGTAGgtaggtactaagatttata 660

IL-2

gtgaaaacatattgtattaagttagtgctcggaattgttcgaagttcaaatctat 715

ttcaagtacttgggttacaacctctcccctcacgaataaatactatacagcttta 770

tgagggacataaaattttcataaatcagtcaatttcaaatttgaataattttaaa 825

taaatctatattttatatatttaaatattctgaacttaatatacaagaccttcat 880

agcgaacctatattatagttctccgaatgcataaattccttgtagagatgcaagg 935

ctccatctcaatctaaatttggagctatattactNNNNNNacgactaccggacta 990

ttgtaggattactctatgtaaacaaatacctacatttatgaacactaatacacta 1045

taaaacataatgtataagtataaacaactaattcaatgcactgtggcaactatcc 1100

cagtgtctgaaaataccctgggatatcctagtgtttttttctatttttaaaaatc 1155

agaaacataaatattgcactgaatatactgagcgattacagtataacaataaata 1210

attcaataataaattaggatgtggttaaattatagtatttaaatgattgtttcag 1265

CAATGAAACACTTGGGAACCATCATCCTGTTTCTATATCTCCTAAGAATGGCTTT 1320

IL-1

CACGTCTCCTGCCATTCAACTGGAAGACGACAGATATATGGATAAGGAGTTTCAG 1375

G3-1

ACGAATGCTGTAAATGCACGAGAGCTTACCAACTGGATAATGCAGATATTAATGC 1430

IR-2

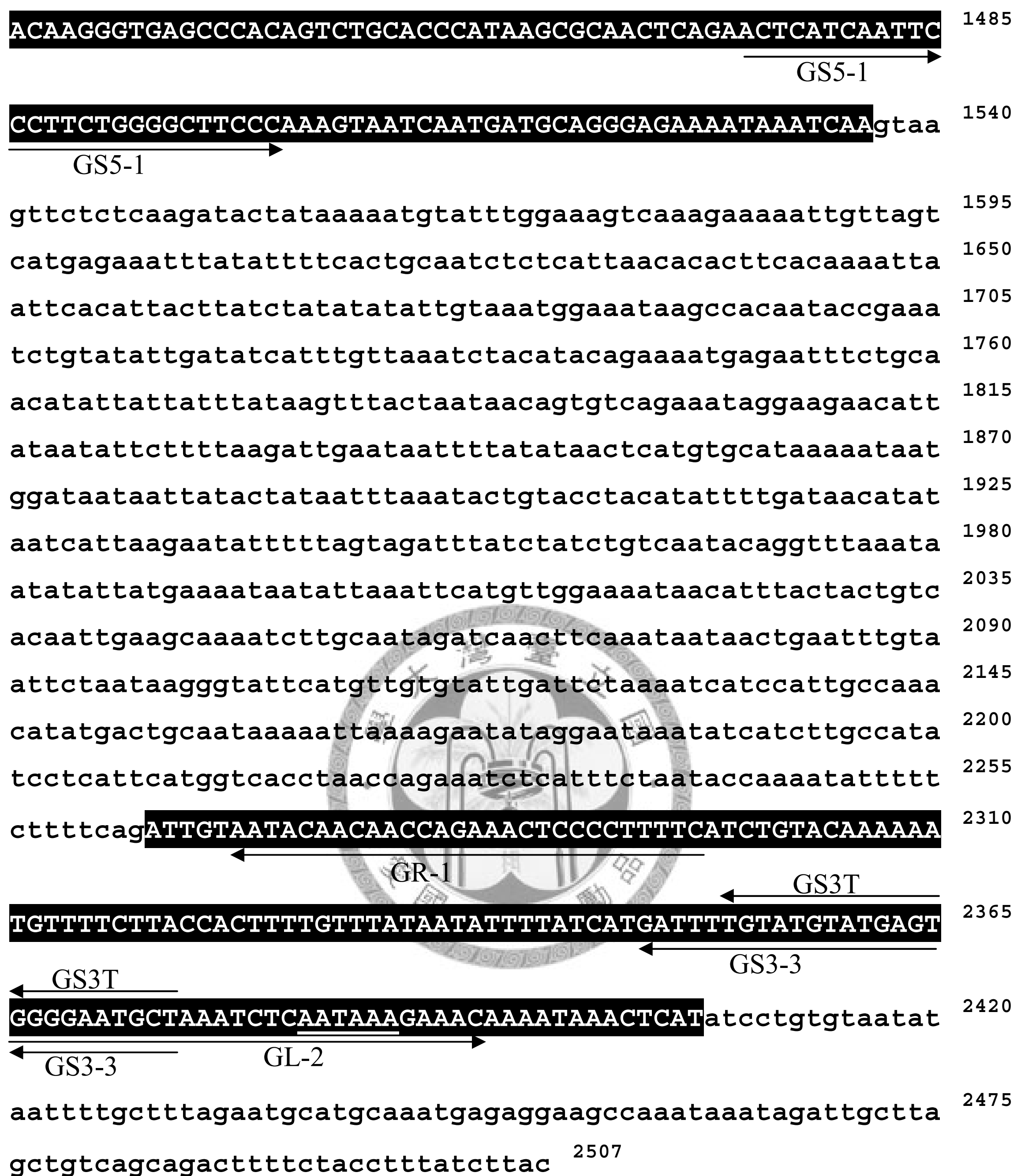

Figure 2-2. The partial nucleotide sequence of the *pdf* gene of the German cockroach. Small letters indicate the genomic sequence and capital letters in black box indicate the cDNA sequence. The capital “N” strand indicates the site of unrevealed sequence. The potential TATA box and a polyadenylation signal are underlined. Primers described in the text are indicated by arrows.

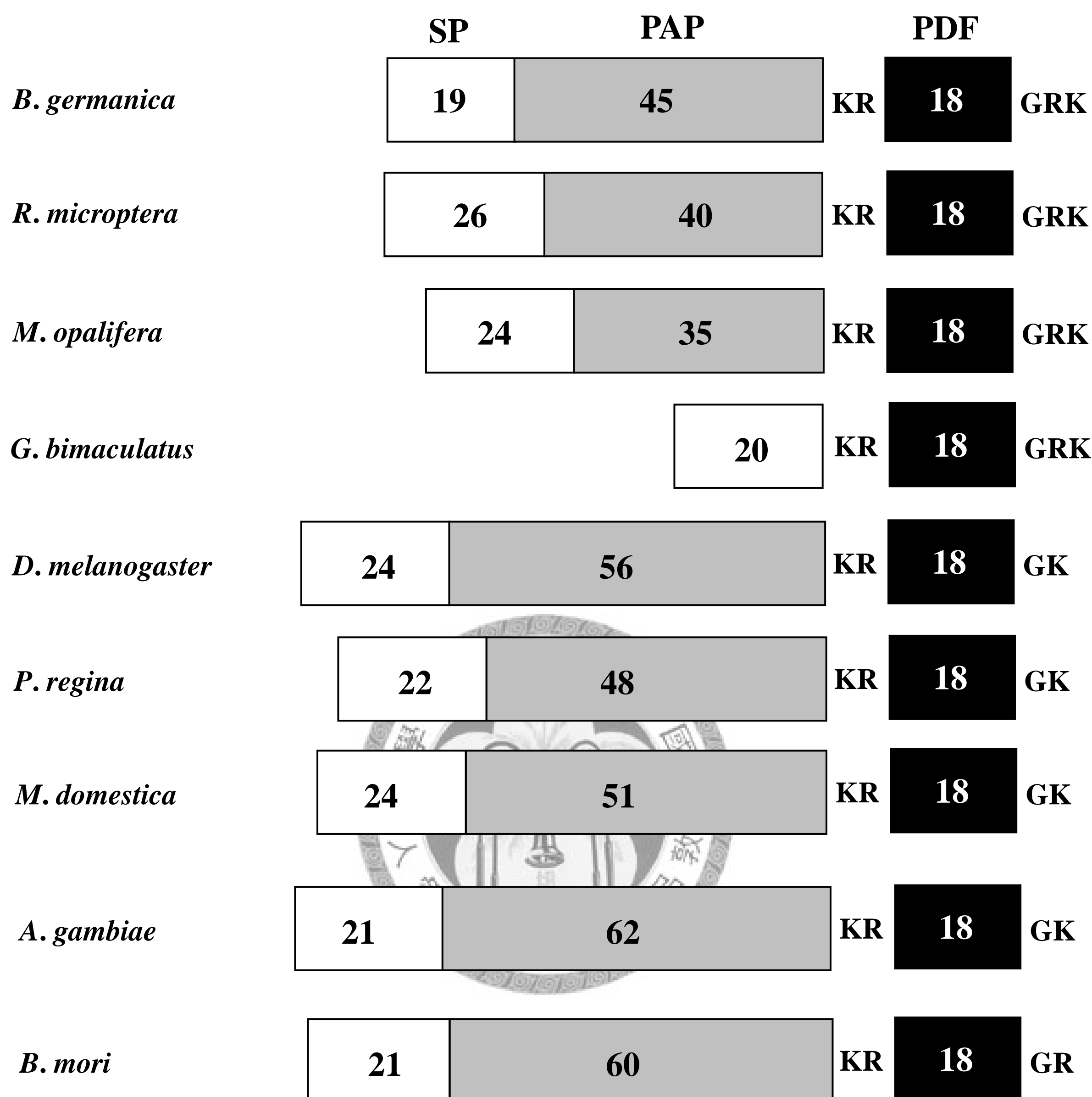

Figure 2-3. The deduced structures of PDF precursor proteins in various insect species. White, gray, and black boxes represent the signal peptide (SP), PDF-associated peptide (PAP), and PDF regions. Each number in the box indicates the number of amino acid residues. The cleavage site (KR) lies between PAP and PDF region. The last 2 or 3 amino acids (GRK, GK, or GR) of the C-terminal are the amidation site.

Meimuna-Romalea group

```
Bg   (87)  M---KHLGTIILFLYLLRMAFTSPAIQLEDDRYM-DK--EFQ-TNAVNAR
Mo   (82)  MRS-A--GVMIAVLAVC---LCLCLESATSLRYQDDKYIESQ--YGPSTR
Rm   (89)  MTAMAVSGKLLTALVLSTYILGLALTIQATQ-YEEDKYQENEVKYG---R

Bg         ELTNWIMQILMHKGEPTVCTHKRNSELINSLLGLPKVINDAGRK
Mo         ELASWLLEWAQ-KND---HAHKRNSEIINSLLGLPKVLNDAGRK
Rm         ELASWLAQLA-HKNEPAICAHKRNSEIINSLLGLPKLLNDAGRK
```

Gryllus group

```
Gb   (43)  MARRARFEANAAPSPLMCVHKRNSEIINSLLGLPKVLNDAGRK
```

Drosophila-Musca group

```
Dm  (102)  MAR-----YTYLVALVLLAICCQWGYCGAMAMPDEERYVRKEYNRDLLD-WFNNVGV
Md   (97)  M-----TNIGYFSLALFWMSLLLCHVATALPAPDEEQYFDKQLNRELINRWLSSIHN
Pr   (92)  MVK-----TLYFLMALVLAAVLVT--VTSLPTPDEERYFDKEFNRDLIN-WLTSIRY

Ag  (105)  MAK--------VSAACVLLVCLWLRASAALPAFEDDRDLDRELYIRQLAEWLADQST
Bm  (103)  MKSVTLLFFLFLMEASTYSVANSEAKIKLNRKVSESSYGSDEQYIRQIHSLVNAYRE

Dm         GQFSPGQVAT-LCRYPLILENS-----LGPSVPIRKRNSELINSLLSLPKNMNDAGK
Md         AQILNN----NPCRFYGGDG--------TWTAPLPKRNSELINSLLSLPKSMNDAGK
Pr         AQPSNN-----PCRYYAGN---------TLTAPMPKRNSELINSLLSLPKNMNDAGK

Ag         DFLNELTSFP-PCRPCSSYEHTRQPIAVVPRAPYAKRNSELINSLLSLPKTMNDAGK
Bm         DNSMLGENFIIETKALIDT-----------KYRTWKRNADLINSLLALPKDMNDAGR
```

Figure 2-4. Multiple sequence alignments of three insect groups of PDF precursor proteins. Numbers in the parentheses represent the length of each PDF precursor. The black background: Residues in that column are identical in all sequences in the alignment. The gray background: Residues in that column are conservative. Ag: *Anopheles gambiae*; Bg: *Blattella germanica*; Bm: *Bombyx mori*; Dm: *Drosophila melanogaster*; Gb: *Gryllus bimaculatus*; Md: *Musca domestica*; Mo: *Meimuna opalifera*; Pr: *Phormia regina;* Rm: *Romalea microptera*.

Gb	NSEIINSLLGLPKVLNDA
Ad	NSEIINSLLGLPKVLNDA
Mo	NSEIINSLLGLPKVLNDA
Rm	NSEIINSLLGLPKLLNDA
Pa	NSELINSLLGLPKVLNDA
Bg	NSELINSLLGLPKVINDA
Ag	NSELINSLLSLPKTMNDA
Dm	NSELINSLLSLPKNMNDA
Pr	NSELINSLLSLPKNMNDA
Md	NSELINSLLSLPKSMNDA
Bm	NADLINSLLALPKDMNDA

Figure 2-5. Multiple sequence alignments of the PDF peptides among 11 insect species. The black background: Residues in that column are identical in all sequences in the alignment. The gray background: Residues in that column are conservative. Ad: *Acheta domesticus*; Ag: *Anopheles gambiae*; Bg: *Blattella germanica*; Bm: *Bombyx mori*; Dm: *Drosophila melanogaster*; Gb: *Gryllus bimaculatus*; Md: *Musca domestica*; Mo: *Meimuna opalifera*; Pa: *Periplaneta americana*; Pr: *Phormia regina;* Rm: *Romalea microptera*.

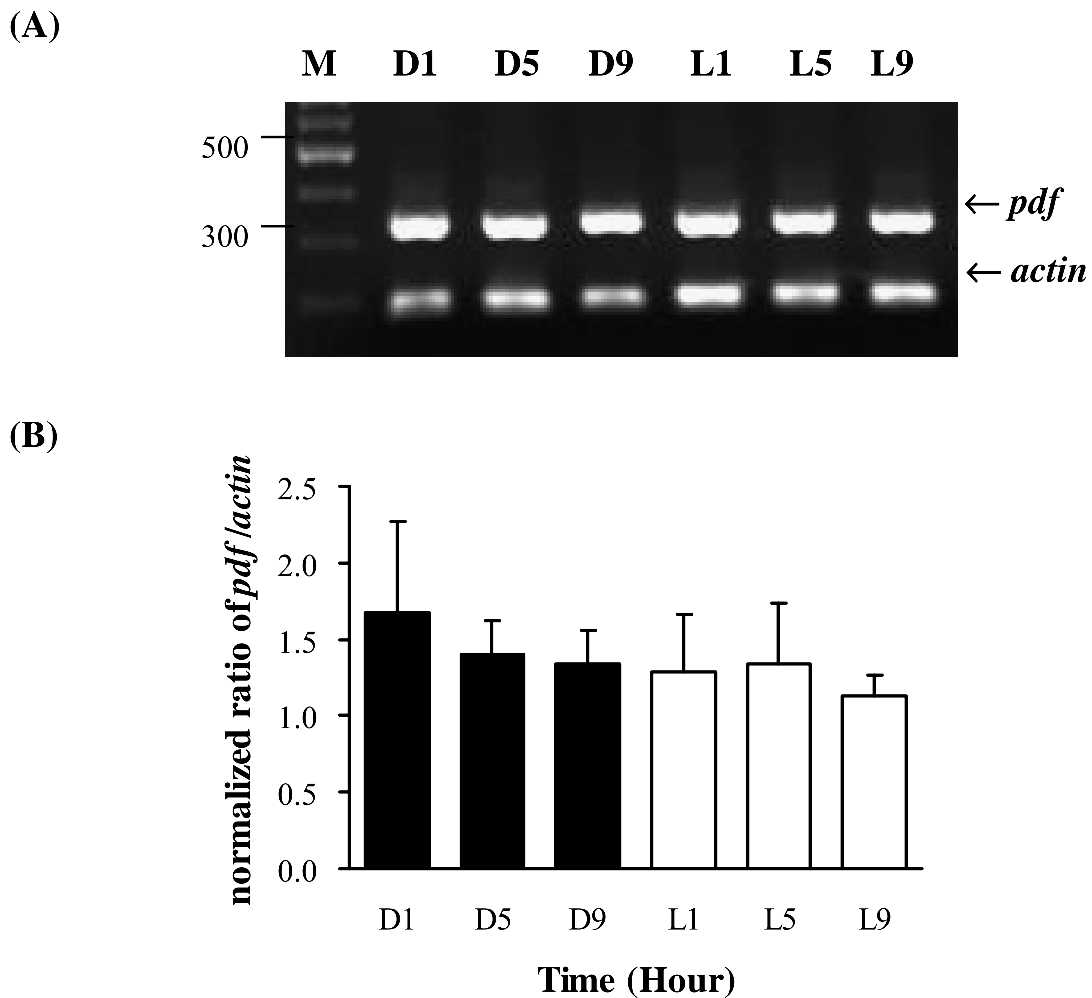

Figure 2-6. The daily expression pattern of *pdf* gene of the German cockroach under light-dark cycle. (A) A representative figure of the result of RT-PCR. Each lane indicates the result of one male head. The symbol above each lane indicates the time of sampling: D and L indicated the dark and light phase, respectively; and the number indicated the hour(s) after light off/on. (B) The averaged quantitative expression of the *pdf* RT-PCR results (n=4). The density of every band in the gels is measured by the program AlphaEase™ (Alpha Innotech). Then, the ratio of *pdf* over *actin* is calculated. For normalization, each ratio is divided by the lowest ratio in each trial. M: DNA marker; *pdf*: partial cDNA of *pdf* mRNA; *actin*: partial cDNA of *actin* mRNA.

Chapter 3

Studying the Functions of the *pdf* Gene in the Circadian Rhythm of the German Cockroach, *Blattella germanica* (L.)

3.1 Introduction

Pigment dispersing factor (PDF) is a neuropeptide identified from insect head extracts (Rao et al., 1991). PDF is composed of 18 amino acids and its sequence is similar to the beta-pigment dispersing hormone (PDH) of crustaceans. Since beta-PDH is responsible for changing body coloration and visual perception in crustacean, PDF in insects would be expected to have different functions because they undergo slow or no body coloration changes in the same developmental stage. From previous immunostaining studies, PDF is synthesized specifically and constantly in the clock cells, which serve as the center for locomotor circadian rhythms of many insects (Homberg *et al.*, 1991; Helfrich-Förster, 1995; Sehadova *et al.*, 2003). We hypothesize that PDF is involved in the control of circadian locomotor rhythms.

The locomotion of *Drosophila* can directly reflect the operation of its circadian clock (Helfrich-Förster, 1998), with a two-peaked locomotor pattern (morning and evening peaks). The pdf^{01} mutant strain could be entrained under the light-dark (LD) cycles, but its evening peak was advanced while the morning peak disappeared. Under constant darkness (DD), the pdf^{01} mutant became arrhythmic in locomotion (Renn *et al.*, 1999). Furthermore, the amount of PDF immunostaining in the dorsomedial terminals of the small ventrolateral neurons (s-LN_vs) changed rhythmically with time of day (Park *et al.*, 2000) and the PDF receptors were located on Dorsal Neurons 1, and 3 (DN1 and DN3) (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005; Shafer et al., 2008). These results indicated that PDF is an output signal, and the circadian clock might use PDF to control the downstream circadian oscillator in the brain. In addition, PDF might also be the synchronizer for coordinating the clock cells. In the pdf^{01} mutant flies, the cycling amplitude of clock gene *tim* mRNA was

gradually lost (Peng *et al*., 2003) and the phase of *per* cytoplasm-nucleus translocation rhythm became dispersed under DD condition (Lin *et al*., 2004).

Several studies have investigated the function of PDF in insect species other than *Drosophila*. In the cockroach *Leucophaea maderae*, the circadian clock is located in the accessory medulla (AMe) of the optic lobe (Sokolove, 1975; Stengl and Homberg, 1994; Reischig and Stengl, 2003). Injecting synthetic PDH in the vicinity of the AMe shifted the onset of circadian locomotor activity in this species (Petri and Stengl, 1997). In addition, PDF locked and synchronized the phase among different AMe cell assemblies in vitro (Schneider and Stengl, 2005). In the cricket *Gryllus bimaculatus*, injecting PDF into the optic lobe evoked an increase in the spontaneous activity of the brain efferents at the optic stalk during the day, but had no effect during the night (Saifullah and Tomioka, 2003). These results suggest that PDF is involved in coordination among cells within a circadian clock and also act as the coupling signal or modulator for the bilateral locomotor circadian clocks.

The German cockroach is a domesticated species with world-wide distribution (Cornwell 1968). Its locomotion is expressed in a circadian rhythm with concentrated activities during scotophase (Dreisig and Nielsen, 1971; Lin and Lee, 1996). The presence of the underlying molecular clock was indicated by the daily fluctuation in expression of the conserved *period* gene (Lin *et al*., 2002). Because the structure of *pdf* gene differs among insect species (Park and Hall, 1998; Chuman et al., 2002; Sato et al., 2002; Matsushima et al., 2004), the functions of PDF might also be diverse among insects. PDF is involved in circadian control system of locomotion in *Drosophila*, cockroach and cricket, but the molecular evidences that support this claim been reported

only for the fruit fly. The RNA interference (RNAi) technique is a very useful tool for studying the functions of the gene in non-model insects. Using RNAi technique, the mRNA of a specific gene would be destroyed soon after transcription, and this gene silencing effect could last for a long period. Since the study of circadian rhythm always last for a long period, RNAi is therefore a powerful tool for investigating the functions of circadian rhythm related gene.

3.2 Materials and methods

3.2.1 Insect

German cockroaches (*Blattella germanica* L.) were reared in an environmental chamber at 28ºC with a 12:12 light/dark (LD 12:12) schedule. Water and food were provided *ad libitum*. To determine the age of experimental cockroaches, adults for the following experiments were collected within 24 hours of emergence and kept in a transparent plastic jar (14 x 14 x 20 cm). Male and female adult cockroaches were separated within 24 hours of eclosion.

3.2.2 RNA extraction

After antennae removing, 5 heads of the male cockroaches were severed and put into 500 µl TRIZOL® reagent (Invitrogen). The sample was homogenized and placed at room temperature for 5 minutes, and then added 100 µl chloroform and shook the mixture vigorously by hand for 15 seconds. Placed the mixture at room temperature for 5 minutes, then centrifuged at 12000g for 15 minutes at 4ºC. Following centrifugation, the upper aqueous phase was transferred into a microtube. An equal volume of isopropanol was added and inverted to mix. Placed the mixture at room temperature for 10 minutes, then centrifuged at 12000g for 10 minutes at 4ºC. Removed the supernatant and Added 500 µl of 75% ethanol to wash the pellet, and then centrifuged at 7500g for 5 minutes at 4ºC. Discarded the supernatant and air-dry the RNA pellet for 5-10 minutes. The RNA pellet was dissolved in 10 µl diethyl pyrocarbonate treated water (DEPC H_2O). The integrity of the total RNA was checked by 0.8% agarose gel electrophoresis. The quality and concentration of the total RNA were determined by the spectrophotometer.

3.2.3 Reverse transcription (RT)

The mRNA was reverse-transcripted into cDNA by Superscript III reverse transcriptase (Invitrogen). At first, 5 µl total RNA , 1 µl 50 µM oligo(dT)$_{20}$ primer, 1 µl 10 mM dNTP Mix, and 3 µl DEPC H_2O were added into a 0.2 ml microtube. Kept the mixture at 65ºC for 5 minutes and then placed it on ice for more than 1 minute. After brief centrifugation, added 2 µl 10X First-Strand Buffer, 4 µl 25 mM $MgCl_2$, 2 µl 0.1 M DTT, 1 µl RNaseOUT™ Recombinant RNase Inhibitor (40 units/µl) and 1 µl of SuperScript™ III reverse transcriptase (200 units/µl). Mixed gently, and then incubated the mixture at 50ºC for 60 minutes, followed by 85ºC for 15 minutes.

3.2.4 Construction of the template for dsRNA synthesis

A 483 bp long *pdf* cDNA fragment was amplified from the product of reverse transcription by PCR. The PCR mixture (25 µl) for amplifying *pdf* fragment included 0.5 µl of reverse transcription product, 10 pmol of GS5T-1 primer, 10 pmol of GS3-3 primer, and 5 µl 5X Taq Master Mix (Protech). PCR (25 cycles) was performed as follows: denaturation at 94ºC for 1 min, annealing at 60ºC for 1 min, extension at 72ºC for 2 min. The GS5T-1 primer was picking from the 5' untranslated region of *pdf* cDNA (base 5-27). The sequence of GS5T-1 primer was 5'- AGTCTGAGAATTTCCTTCT CGACC -3'. The GS3-3 primer was picking from the 3' untranslated region of *pdf* cDNA (base 450-483). The sequence of GS3-3 primer was 5'- AGCATTCCCCACTCA TACATACAAAATC -3'.

The amplified DNA molecules of 75 µl PCR product were purified from the gel by using DNA extract kit (Viogene), and were dissolved in 15 µl H_2O. The purified DNA molecules were cloned into the pGEMTeasy vector (Promega), and transformed into the *E. coli*.

3.2.5 RNA Interference

The *pdf* double-stranded RNA (dsRNA) was synthesized by PCR-template method (Kennerdell and Carthew, 1998). The primers which contained the sequence of T7 promoter and gene specific part were used in this PCR reaction. The sequence of T7 promoter was TAATACGACTCACTATAGGGAGACCAC and located in the 5' end of the primer. The sequences and locations of gene specific part of primers (GS5T and GS3T) were indicated in the Figure 2.

The PCR mixture (25 μl) included 1μl of 1/5000X plasmid, 10 pmol of each primers, and 5 μl 5X Taq Master Mix (Protech). PCR was performed in two stages. In the first stage, the reaction processed for 6 cycles: denaturation at 94ºC for 1 min, annealing at 60ºC for 1 min, extension at 72ºC for 1 min. In the second stage, the reaction processed for 19 cycles: denaturation at 94ºC for 1 min, annealing at 70ºC for 1 min, extension at 72ºC for 1 min. The PCR product was concentrated by isopropanol precipitation.

The in vitro transcription reaction was performed using MEGAscript® RNAi kit (Ambion). The reaction was done according the instruction of the kit. Three microgram template was used for one reaction. The product of in vitro transcription was treated with DNase I and RNase to digest the dsDNA template and ssRNA, and cleaned up by spin column method. The *pdf* dsRNA was eluted from the column by DEPC H_2O.

One microgram dsRNA was injected into the abdomen of each cockroach to evoke the RNA interference effect. For the control group, each cockroach was injected with equal volume of DEPC H_2O. After injection, each cockroach was put into the rearing container or locomotion detector box immediately.

3.2.6 RT-PCR

The effect of dsRNA injection was checked by RT-PCR. For RT-PCR, the heads of control and dsRNA-injected cockroaches were severed at various days after injection. The total RNA of each head was extracted with TRIZOL® reagent (Invitrogen). The RNA pellet was dissolved in 10 μl DEPC H_2O. Eight microliter RNA was reverse transcribed by using Superscript III reverse transcriptase (Invitrogen) and the oligo$(dT)_{20}$ primer was used. The PCR mixture (25 μl) for amplifying *pdf* fragment included 1 μl of reverse transcription product, 10 pmol of forward primer (5'-CTGCCATTCAACTGGAAGAC GA-3'), 10 pmol of reverse primer (5'-AGCATTCCCCACTCATACATACAAAATC-3'), and 5 μl 5X Taq Master Mix (Protech). PCR (25 cycles) was performed as follows: denaturation at 94ºC for 1 min, annealing at 60ºC for 1 min, extension at 72ºC for 1 min. Beside, PCR for amplifying *actin* fragment had also been performed. The PCR mixture (25 μl) included 1 μl of 10 times diluted reverse transcription product, 10 pmol of forward primer, 10 pmol of reverse primer, and 5 μl 5X Taq Master Mix (Protech). The PCR program was described as above. The PCR products were identified on a 1.5% agarose gel.

3.2.7 Immunostaining

The immunostaining was performed as described previously (Sehadova et al., 2003; Wen and Lee, 2008). The PDF peptide of the German cockroach and anti-PDF polyclonal antibody were synthesized by BIOMEN (Taiwan). The sequence of PDF (NSELINSLLGLPKVINDA) is predicated from the corresponding cDNA sequence of the German cockroach. The brain of the cockroach was dissected and fixed in 4% para-formaldehyde in phosphate buffer saline (PBS) overnight at 4ºC. The brain was washed in PBST (1% Triton X-100 in PBS) at room temperature for 10 min and

repeated for 3 times. The brain was treated with collagenase at room temperature for 30 min and followed by treating with methanol series: 50%, 70%, 100%, 70%, and 50%. The brain was kept in each concentration at room temperature for 10 min. After two times PBST washing (room temperature, 10 min for each time), the brain was kept in the 10% normal goat serum in PBST at room temperature for 2 hours and followed by treating with primary antiserum for 48 hours at 4ºC. The primary (anti-PDF) antiserum was diluted 1:300 in PBST. After primary antiserum treatment, the brain was washed threes times with PBST at room temperature. The brain was then kept in the secondary antibody solution for 24 hours at 4ºC. The goat anti-mouse IgG with conjugated fluorescein isothiocyanate (KPL) was used as secondary antibody. The secondary antibody was diluted 1:100 in PBST. The brain was washed 3 times with PBST and once with H_2O at room temperature. The brain was transferred into 50% glycerin, and checked with flourorecence microscope. The images of a brain sample from different focal planes were combined by software Helicon Focus (Helicon Soft).

3.2.8 Locomotor activity monitoring

The locomotor activity of the cockroach was monitored by an automatic locomotion detecting system under 28ºC and LD 12:12 or DD condition. Each cockroach was allowed to run in a transparent Plexiglas box (24 x 2.5 x 3cm) with six pairs of infra-red sensors, and this box was placed in an automatic motion detector system. When the cockroach moved and blocked the infra-red beam, this event was automatically registered as an activity. Other detailed information about this data acquisition system was described in a previous report (Lin and Lee, 1998). The rhythmicity of daily locomotion and the circadian period were analyzed by chi-square periodogram (Sokolove and Bushell, 1978). The

cockroach was statistically determined as rhythmic when the height of the periodogram peak was larger than $1.2\chi^2$ and the width of the periodgram peak was wider than 0.7 hour (Reischig and Stengl, 2003).

3.3 Results

3.3.1 Effects of *pdf* dsRNA injection on the *pdf* gene expression in the transcription level

The *pdf* gene expression in the German cockroach was suppressed by injection of dsRNA (Fig. 3-1). The silencing effect detected by RT-PCR occurred 2 days after treatment. Although some treated individuals still expressed *pdf* RNA weakly at 3rd day after injection, no more expression was detected afterwards, and the silencing effect persisted at least for 56 days (Fig. 3-1). When the samples from different time points within one day were checked by RT-PCR, no *pdf* cDNA could be detected at any time points (Fig. 3-2).

3.3.2 Effects of *pdf* dsRNA injection on the PDF peptide synthesis

The effect of *pdf* dsRNA injection has also been checked in the protein level by using immunostaining technique. In the normal male cockroach, three groups of PDF-immunoreactive (PDF-ir) cells could be identified in the optic lobe (Fig. 3-3). The PDF-ir cells that stained most strongly were located in the proximal frontoventral region of the accessory medulla (PDF-AMe). There were also two groups of lightly stained cells in the posterodorsal and posteroventral region of the distal medulla (PDF-pddMe, and PDF-pvdMe) could sometimes be stained. The neural processes of PDF-ir cells extended over the optic lobe in a fan shape. In addition, the PDF-ir cells also sent neural processes to the protocerebrum and contralateral side of the brain. The anti-PDF immunostaining result of the female cockroach was similar to that of the male cockroach (Fig. 3-5).

The suppression effect of *pdf* RNAi was reflected by the gradually weakening intensity of stain in the cell bodies of PDF-AMe since the fifth day after injection (Fig.

3-4). On the tenth day, the cell bodies of PDF-AMe could only be weakly stained in the male cockroach, but they were not stained on the thirty-sixth and fifty-sixth days after injection. Furthermore, the fan-shaped neural processes in the optic lobe became weakly and partially stained since the fifth day after injection, and their terminals could still be weakly stained on the fifty-sixth day after injection. As for the neural processes extending to the protocerebrum and the contralateral side of the brain, their staining intensity weakened substantially on the fifth day after injection, and staining was not detected since then. In the female cockroach, both the cell bodies and the neural processes could not be stained at the tenth day after dsRNA injection (Fig. 3-5A).

3.3.3 Effects of *pdf* gene silencing on circadian locomotion

Under LD conditions, untreated male cockroaches were entrained and showed a nocturnal activity pattern (Fig. 3-6B). However, injection of *pdf* dsRNA disrupted the expression of locomotor circadian rhythm in experimental males (Fig. 3-6A). On the 20th day after injection, 47.8% of the treated cockroaches (n= 23) became arrhythmic, but all the control cockroaches (n= 13) remained rhythmic in locomotion (Table 3-1 and Fig. 3-6). This result indicated the treated cockroaches lost their ability to synchronize with the Zeitgeber due to silencing their *pdf* gene.

Under DD condition, male cockroach showed a free-running rhythm with a circadian period shorter than 24 hours, so that the light-off activity peak appeared earlier than the previous day (Fig. 3-7B). The effect of silencing the *pdf* gene appeared in 87.5% of the treated cockroaches (n= 16) who became arrhythmic 20 days after injection, while only 11.1% of the sham control (n= 9) lost their freerunning rhythm (Table 3-1 and Fig. 3-7). Beside, when the lighting condition change from DD into LD again, 85.7% of the *pdf* dsRNA injected cockroaches (n= 7) could not be entrained by

the LD cycles, while only 17.7% of the control group (n= 6) could not be entrained (Fig. 3-8).

Not only was the circadian locomotor rhythm disrupted by *pdf* dsRNA injection, but the quantity and pattern of locomotion were also significantly affected. Injection of *pdf* dsRNA caused a significant decrease in daily locomotor activity (Fig. 3-9). Since the cockroach is nocturnal, daily activity was concentrated in the scotophase and the activity peak appeared soon after light off. After *pdf* dsRNA injection, the activity peak could not be seen on the 20 days after injection both in LD and DD conditions (Fig. 3-9). When analyzed further, the records from 1-10 days after injection revealed that the size of the activity peak gradually diminished since the second day after injection, and could be distinguished since the 7th day after injection (Fig. 3-10). These results implied that PDF was involved in the regulation of locomotion and expression of its circadian rhythm.

The locomotion of treated female cockroach had also been analyzed. For being masking by some factors, the actogram of the female cockroach was quite different from that of the male cockroach (Fig. 3-11). The activity of female cockroach did not concentrate in the scotophase. During the ootheca-carrying stage, the activity was low for all day long (gap stage in Figure 3-11). After the ootheca dropping, the reproduction cycle stared and the locomotor activity increased significantly. The locomotor activity was kept in a high level for all day long until the new ootheca formed (active stage in Figure 3-11). Injecting *pdf* dsRNA did not change the pattern of locomotion (Fig. 3-11A). The durations of active and gap stages did not change significantly after injection (Fig. 3-12). The daily activity of active and gap stages of experimental and control groups were also similar (Fig. 3-13). These results suggested that PDF did not involve in the masking mechanism of the female cockroach.

3.3.4 Effects of *pdf* dsRNA injection on the reproduction of the female German cockroach

For revealing whether the *pdf* dsRNA injection has influences on reproduction. The treated females were mate with untreated males. Ootheca formation, number of 1st instar nymphs, and ratio of nymphs that could molt into adults were used as the indexes. After *pdf* dsRNA injection, the percentage of experimental group females that could form ootheca was 81% (n= 21; Table 3-2). Although the percentage was lower than the control group (90.9%, n= 33), but it was still a high percentage. The averaged number of 1st instar nymphs and the ratio of nymphs that could molt into adults were not significantly different from control group (Table 3-2).

3.4 Discussion

Locomotion of the German cockroach displays a robust circadian rhythm, so the underlying mechanism is expected to be involved in clock gene expression and time signal transduction. Mutation of clock genes is a powerful tool to investigate the mechanism (Renn et al., 1999; Hyun et al., 2005). However, the lack of genetic tools is a common disadvantage in study molecular mechanism of behavior in non-model animals. In this study, we silenced the expression of *pdf* gene in adult cockroaches. This silencing effect on mRNA levels began on the second day of treatment, and the PDF levels in the pacemaker neurons started to fall after 5 days. The silencing effect persisted for a long time (> 56 days, equaled to a half of male adult's lifespan). With this long-lasting effect, we monitored the locomotor behavior in PDF-deficient male cockroaches for a prolonged period.

After *pdf* dsRNA injection, about 50% of the cockroaches became arrhythmic in LD condition and up to 90% in DD. Therefore, we could expect that PDF plays an important role in the control of locomotor circadian rhythm. This finding, however, is different from the results of studies on *Drosophila*. In *Drosophila*, *pdf* mutant and *han* (PDF receptor) mutant strains are arrhythmic only under DD condition, while they are rhythmic under the LD condition (Renn et al., 1999; Hyun et al., 2005). Therefore, we suggest that the specific roles of PDF differ between the fruit fly and the cockroach.

The German cockroach increased its daily total activity along with age to cope with the stress on reproduction from isolation. The *pdf*-silenced adults did not increase daily total activity, to the contrary, total daily activity decreased (Table 3-1). When we further analyzed the activity pattern, the decrease in daily total activity was mainly due to the disappearance of activity peak at light-off. These concentrated activities were gradually decreased since the secondary day after dsRNA injection, and then could not

be distinguished on the 7th day after injection (Fig. 3-10). In addition, the PDF was predicted as a secretory peptide. Based on these results, we inferred that PDF might serve as the output signal from the pacemaker cells to regulate the control center of locomotion. Without PDF, the output signal cascade of pacemaker is disrupted so that the cockroaches could not start their locomotor activity at the beginning of night or subjective night on each day. In *Drosophila*, PDF also plays an important role in triggering the start of activity under DD and for morning activity under LD conditions (Renn et al., 1999). In *Drosophila*, locomotion is controlled by two groups of cells, the morning and evening cells (Stoleru et al., 2004; Grima et al., 2004; Rieger et al., 2006), and the evening cells do not express PDF. Although PDF plays an important role in triggering the start of activity under DD and for morning activity under LD conditions (Renn et al., 1999), *Drosophila pdf* mutants are rhythmic under LD condition and the daily activity of the similar to that of wild type flies (Renn et al., 1999). These contrasting results imply that PDF is the only output factor involved in the control of locomotor neural circuitry in the German cockroach, whereas the fruit fly should have molecule(s) other than PDF to act as an output signal(s) for circadian locomotion.

The locomotor circadian clock of the German cockroach was suggested to locate in the optic lobe, because the male cockroaches lost their locomotor circadian rhythm after both sides of the optic tracts have been severed (Wen and Lee, 2000). But which area of optic lobe was the possible location for the circadian clock had not been decided. According to the anti-PDF immunostaining, the *pdf* gene might have expressed in three groups of cells in the optic lobe. The PDF-pddMe and PDF-pvdMe cells in the distal medulla were lightly stained by the anti-PDF antibody, and this weak immunoreactivity did not disappear at dsRNA injection (Fig. 3-4). So these two groups of cells might be not the candidates of the circadian clock. The PDF-AMe cells were located in the

accessory medulla. They expressed high anti-PDF immunoreactivity, and this immunoreactivity disappeared after *pdf* dsRNA injection. By comparing the results of immunostaining and locomotion, as the anti-PDF immunoreactivity of the PDF-AMe cells started to fall, the activity peak of the locomotion was also started to weaken (Fig. 3-4, 3-10). Based on these results, the PDF-AMe cells were the most possible candidate of locomotor circadian.

In another cockroach, *L. maderae*, pacemaker cells projected into the contralateral optic lobe with axons and this process was necessary for the synchronization of bilateral pacemakers (Stengl and Homberg, 1994; Reischig and Stengl, 2003). In the German cockroach, the pacemakers also send projections into the contralateral optic lobes (Wen and Lee, 2008). After injection of *pdf* dsRNA, the staining intensity of neural processes extending to the contralateral sides of the brain decreased gradually and could not be stained on the sixth day onwards (Fig. 3-4). In the meanwhile, the cockroaches lost their activity rhythm of locomotion. According to this result, we infer that PDF might also be the synchronizer for bilateral pacemakers in the German cockroach.

In the normal female, its locomotor activity was very low during the ootheca-carrying stage. After the ootheca dropped and the ovarian development started, the locomotor activity started to increase and kept at high level all day long for several days (Lee and Wu, 1994). The female cockroaches which developed from ovary-removed nymphs expressed a different activity pattern; their activity pattern was similar to the male cockroach (Lin and Lee, 1996). These results revealed that the locomotor activity of the female cockroach was coincided with its reproduction. In my studies, neither the reproduction nor the locomotion of the female cockroach has been changed by *pdf* dsRNA injection. Therefore, PDF might not be involved in the

regulation of reproduction of the female cockroach, so could not remove the masking effect.

Table 3-1. The effects of *pdf* gene knockdown on locomotor circadian rhythm of male German cockroaches

Lighting condition	Treatment (injection with)	n1	Circadian rhythm expressed (%)		Circadian period (mean±SE)		Daily locomotor activity (mean±SE)[a]		
			10 days before injection	11-20 days after injection	10 days before injection	11-20 days after injection (n2)	10 days before injection (n1-n2)	11-20 days after injection (n1-n2)	Paired *t*-test[b]
LD	*pdf* dsRNA	23	100	52.2	24.0±0.1	24.0±0.2 (12)	168.8±7.4 (11)	153.9±22.4 (11)	$p>0.05$
	DEPC H_2O	13	100	100	24.0±0.1	24.0±0.1	203.3±18.7	336.2±28.8	$p<0.001$
DD	*pdf* dsRNA	16	100	12.5	23.5±0.4	23.9±0.1 (2)	235.4±32.7 (14)	203.4±38.2 (14)	$p>0.05$
	DEPC H_2O	9	100	88.9	23.6±0.4	23.4±0.3 (8)	158.3±29.8 (8)	184.5±21.3 (8)	$p<0.05$

[a] Daily locomotor activity was counted as number of disruptions of infrared sensors within one day before or after injection.

[b] Paired *t*-test was used to compare the daily activity before and after the injection.

Table 3-2. The effects of *pdf* gene silencing on the reproduction of the female German cockroach

	Percentage of females could form ootheca (n)	Number of nymphs hatching from one ootheca	Percentage of nymphs could grow into adults
Experimental group	81.0 % (17/21)	33 ± 8	70.5%
Control group	90.9 % (30/33)	34 ± 5	70.5%

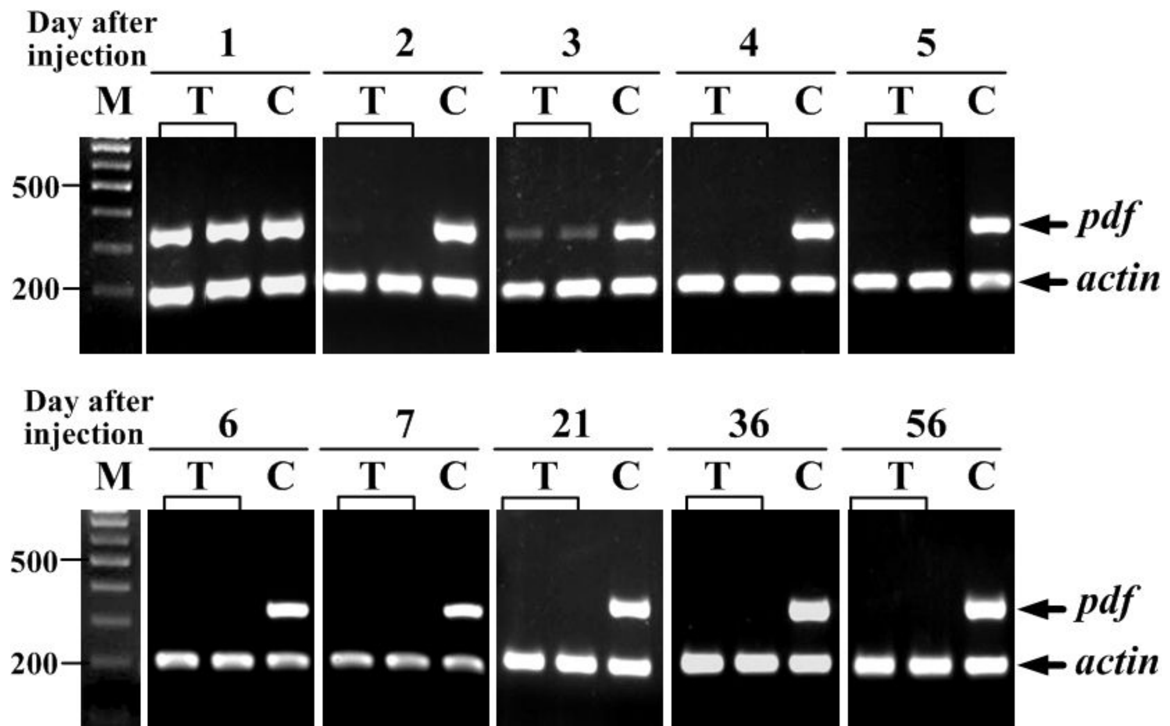

Figure 3-1. The RNA expression pattern in the head of male the German cockroach after *pdf* RNA interference treatment. The total RNA of the head is extracted and then amplified by RT-PCR. M: DNA marker; T: *pdf* dsRNA injection; C: DEPC H_2O injection; *pdf*: partial cDNA of *pdf* mRNA; *actin*: partial cDNA of *actin* mRNA.

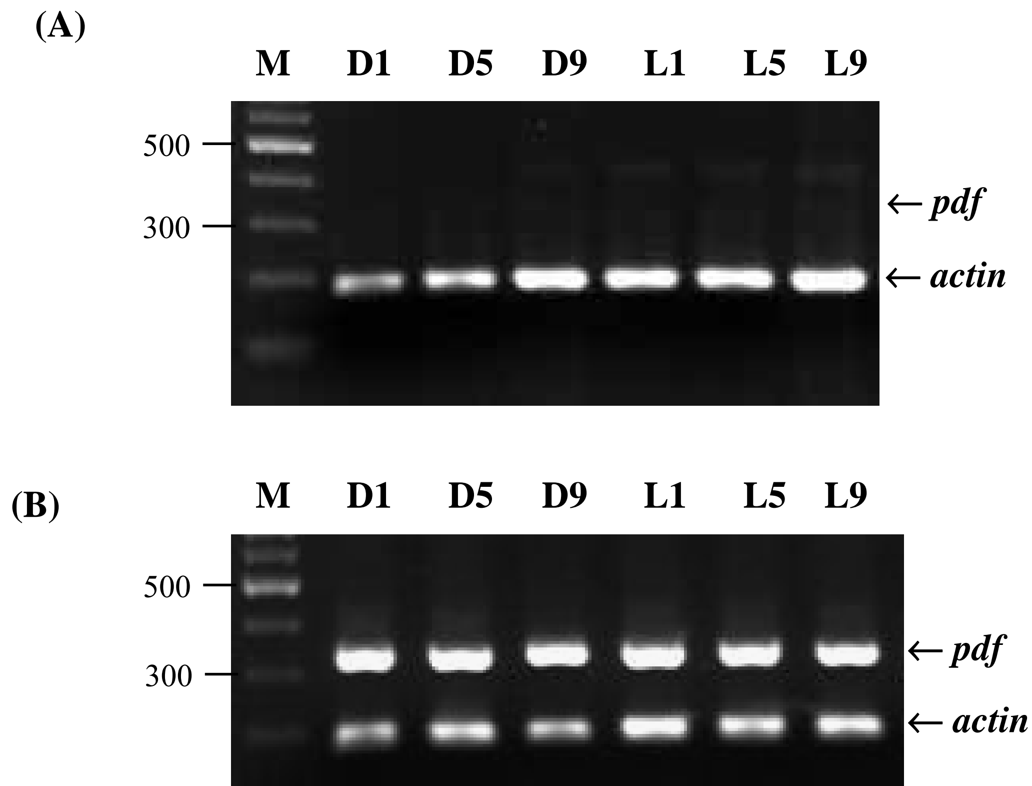

Figure 3-2. The *pdf* gene expression pattern of the male cockroaches on the tenth day after injection under light-dark cycle. Each lane indicates the result of one male head. (A) A representative figure that shows the result of RT-PCR of *pdf* dsRNA injected males. (B) A representative figure that shows the result of RT-PCR of DEPC H_2O injected males. The symbol above each lane indicates the time of sampling: D and L indicated the dark and light phase, respectively; and the number indicated the hour(s) after light off/on. M: DNA marker; *pdf*: partial cDNA of *pdf* mRNA; *actin*: partial cDNA of *actin* mRNA.

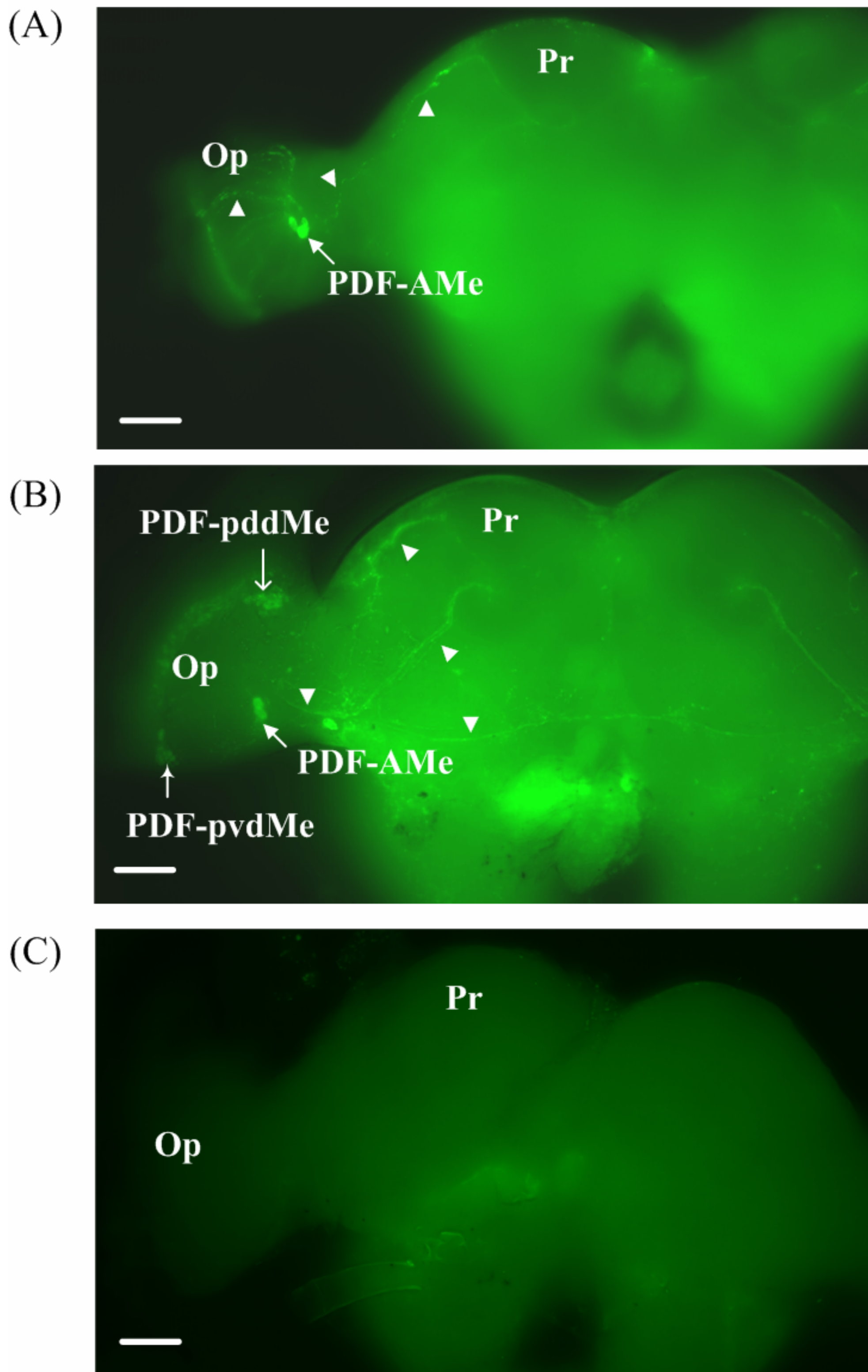

Figure 3-3. The PDF-immunoreactive (PDF-ir) cells in the brain and optic lobes of the male German cockroach. Sample was treated with anti-PDF antibody (A, B) or PBST buffer (C). Pictures were taken from the frontal side (A) and posterior side (B, C). The

cell bodies of three PDF-ir cell groups are pointed by three types of arrow and the projections of the PDF-ir cells are indicated by the arrow heads. Pr: protocerebrum; Op: optic lobe; PDF-AMe: PDF-ir cells in the proximal frontoventral region of the accessory medulla; PDF-pddMe: PDF-ir cells in the posterodorsal region of the distal medulla; PDF-pvdMe: PDF-ir cells in the posteroventral region of the distal medulla. Scale bar = 100 μm.

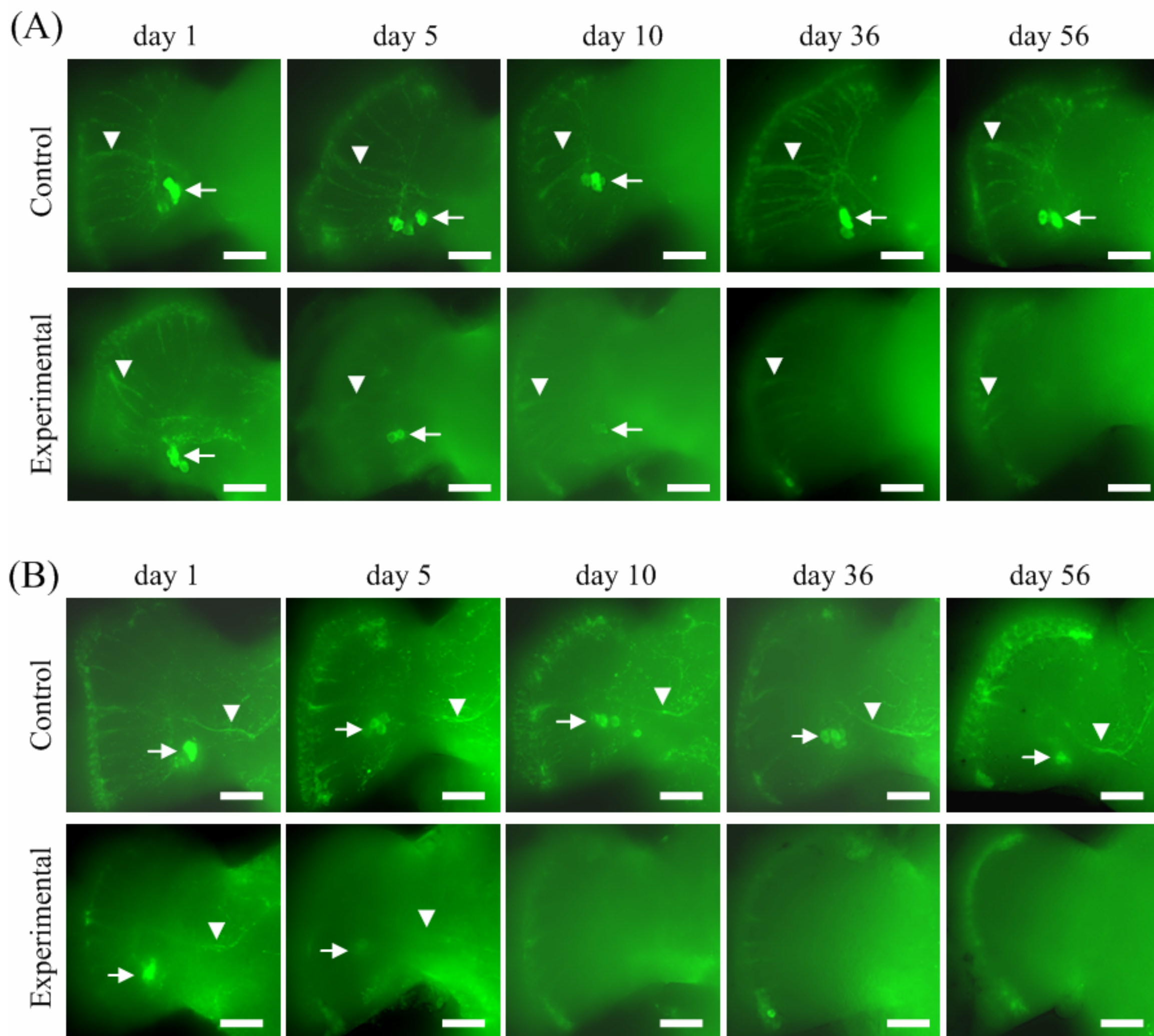

Figure 3-4. The pattern of PDF immunoreactivity of the optic lobes of the male German cockroach after RNAi treatment. The comparison of PDF immunoreactive (PDF-ir) cells and projections between treated and shamed control cockroaches at 1, 5, 10, 36, and 56 days after injection are shown in frontal (A) and posterior view (B). The cell bodies of the PDF-ir cells in the proximal frontoventral region of the accessory medulla (PDF-AMe) are pointed by the arrow, and the arrow heads indicate the projections of the PDF-ir cells.. The days after injection are shown above each picture. Scale bar = 100 μm.

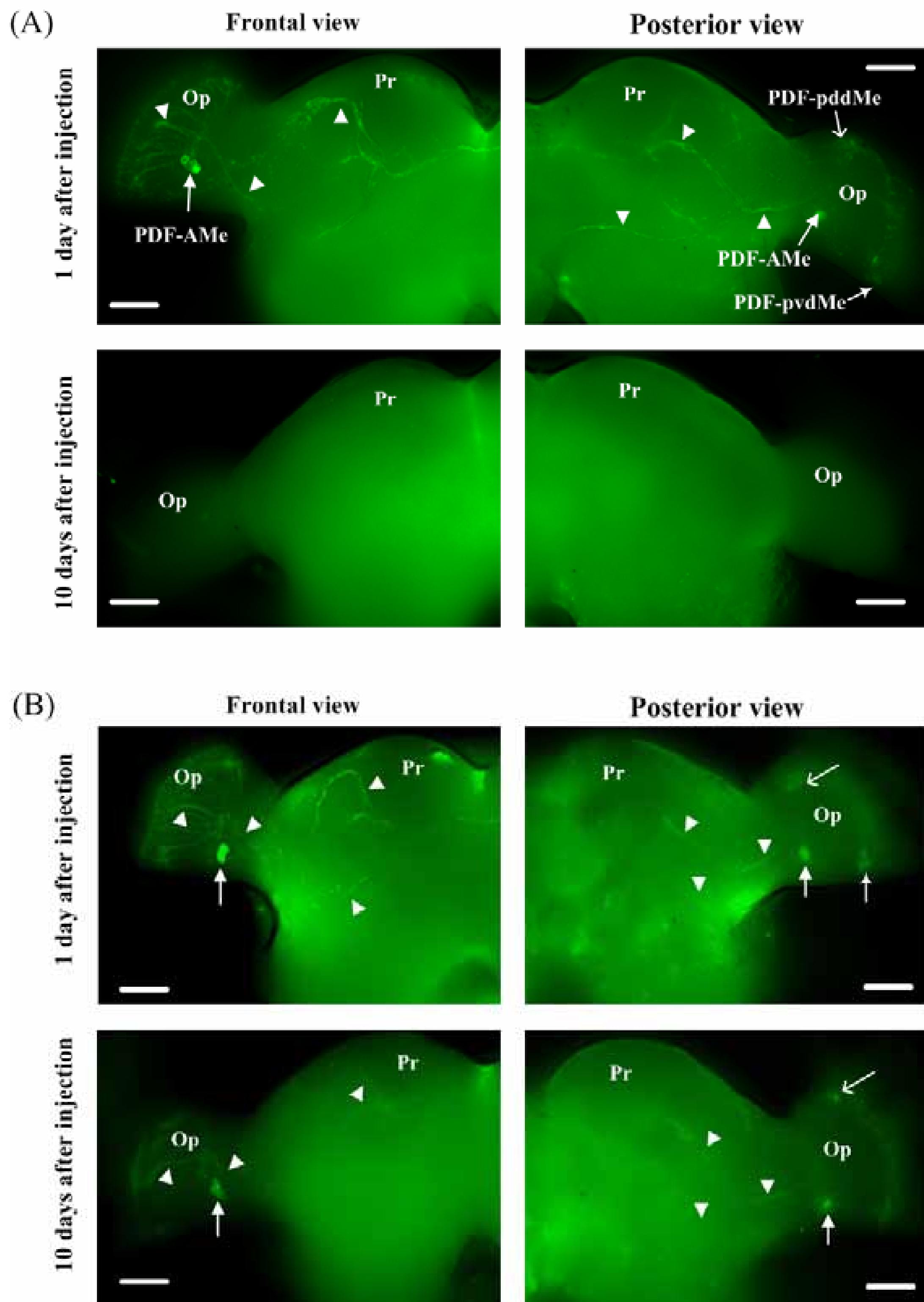

Figure 3-5. The PDF-immunoreactive cells in the brain and optic lobe of the female German cockroach. (A) Representative photographs of the experimental group. The cockroaches are sampled at the first and tenth days after *pdf* dsRNA injection. (B)

Representative photographs of the experimental group. The cell bodies of three PDF immunoreactive (PDF-ir) cell groups are pointed by three types of arrow. The arrow head indicates the projections of the PDF-ir cells. Pr: protocerebrum; Op: optic lobe; PDF-AMe: PDF-ir cells in the proximal frontoventral region of the accessory medulla; PDF-pddMe: PDF-ir cells in the posterodorsal region of the distal medulla; PDF-pvdMe: PDF-ir cells in the posteroventral region of the distal medulla. Scale bar =100 μm.

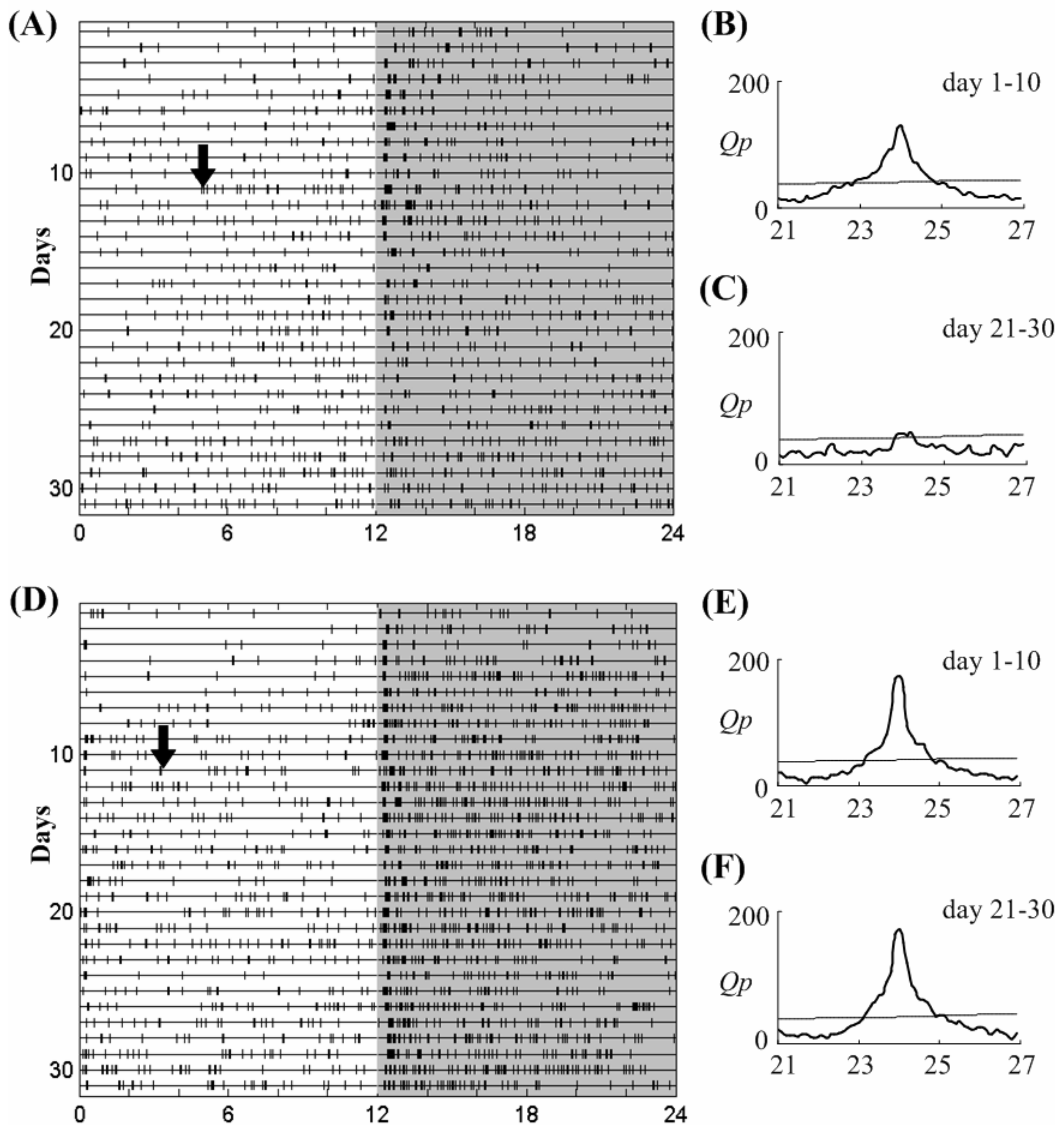

Figure 3-6. Patterns of the locomotor activity in the male German cockroaches from *pdf* RNAi experimental (A-C) and control (D-E) groups under light-dark cycles (LD = 12:12h). The gray background on the actogram (A and D) indicates the dark condition of the environment, while the white background indicates the light phase. The arrow on the actogram indicates the time point of injection. The peridograms of two sections under the DD condition are shown in (B) (E) and (C) (F) for 10 days before and after injection, respectively.

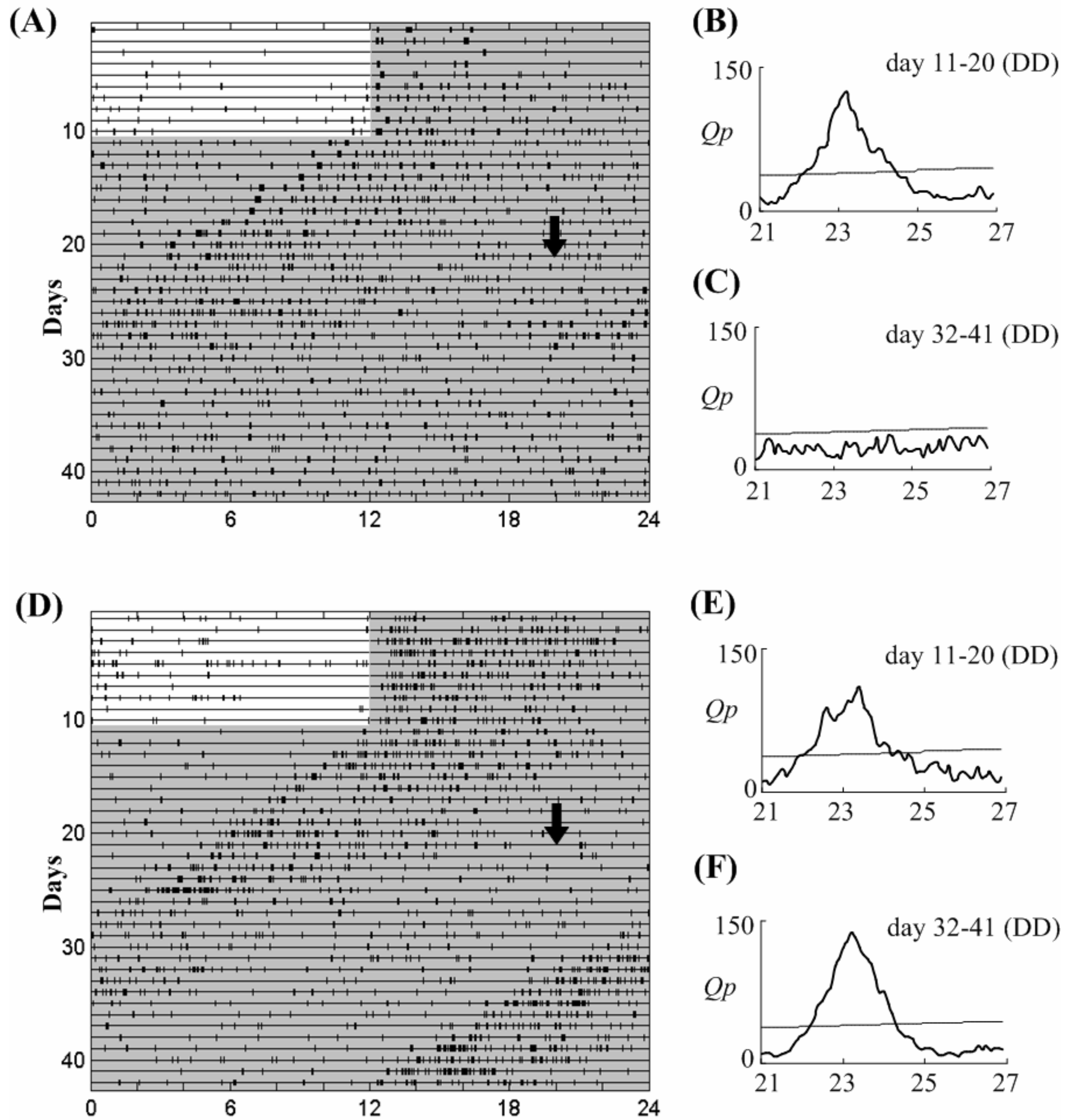

Figure 3-7. Locomotor activity patterns of the male German cockroaches from *pdf* RNAi experimental (A-C) and control (D-F) groups under constant darkness condition. The gray background on the actogram (A and D) indicates the dark condition of the environment, while the white background indicates the light phase. The arrow on the actogram indicates the time point of injection. The peridograms of two sections under the DD condition are shown in (B) (E) and (C) (F) for 10 days before and after injection, respectively.

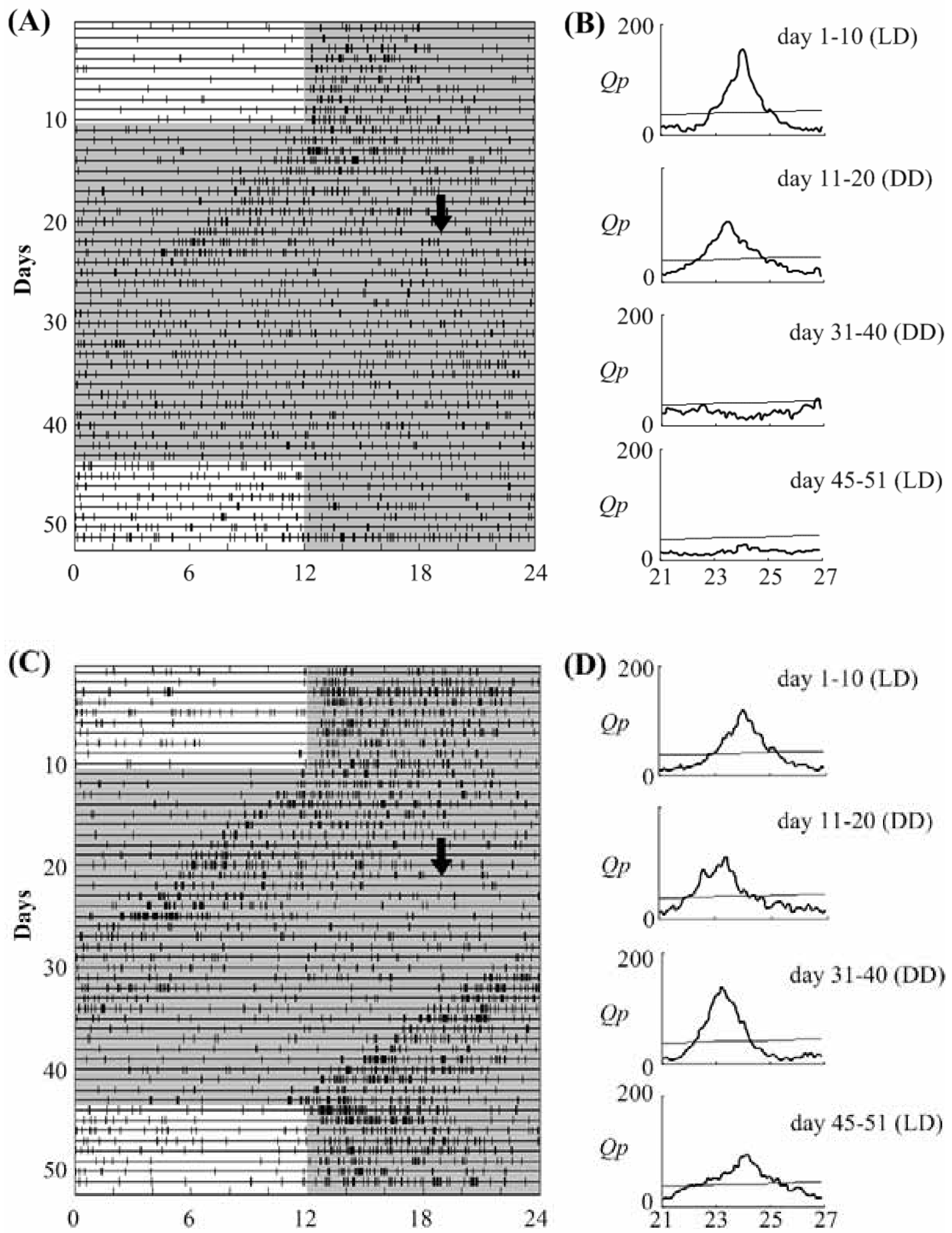

Figure 3-8. Locomotor activity patterns of the male German cockroaches from *pdf* RNAi experimental (A, B) and control (C, D) groups under shift of light condition. The gray background on the actogram (A and D) indicates the dark condition of the

environment, while the white background indicates the light phase. The cockroaches were first entrained with 10 light-dark cycles (LD = 12:12h), then the lighting condition turned into constant darkness condition (DD). After staying at DD for 33 days, the lighting condition turned into LD. The arrow on the actogram indicates the time point of injection. The peridograms of 4 sections under the LD and DD conditions are shown in (B) and (C).

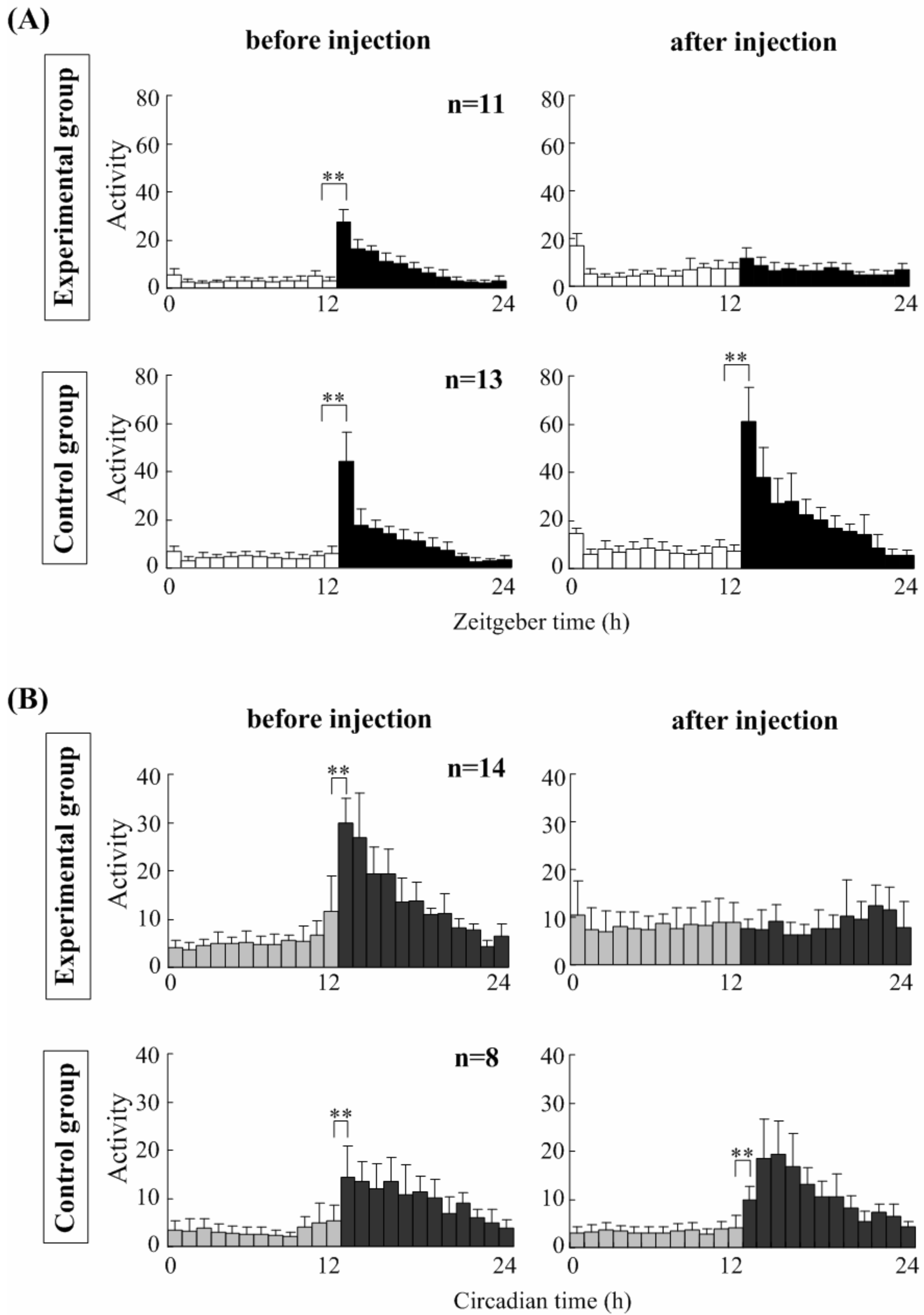

Figure 3-9. The distribution pattern of locomotor activity of the male German cockroach. The activity is the average of 10 days records. “Before injection” represents

the data of 1-10 days before injection, and “after injection” represents the data of 11-20 days after injection. (A) The cockroaches are kept under a light-dark cycle (LD = 12:12h). (B) The cockroaches are kept in the constant darkness environment. Each bar in the histogram represents 1 hour of cumulative activities. The white bar indicates the day and the black bar indicates the night; the gray bar indicates the subjective day and the dark gray bar indicates the subjective night. Double asterisks (**) denotes average activity of the hour 13 (first hour of the night) different from the average activity of hour 12 (last hour of the day) at the level of $p < 0.01$ (Student’s *t*-test). Control group: DEPC H_2O injection; Experimental group: *pdf* dsRNA injection.

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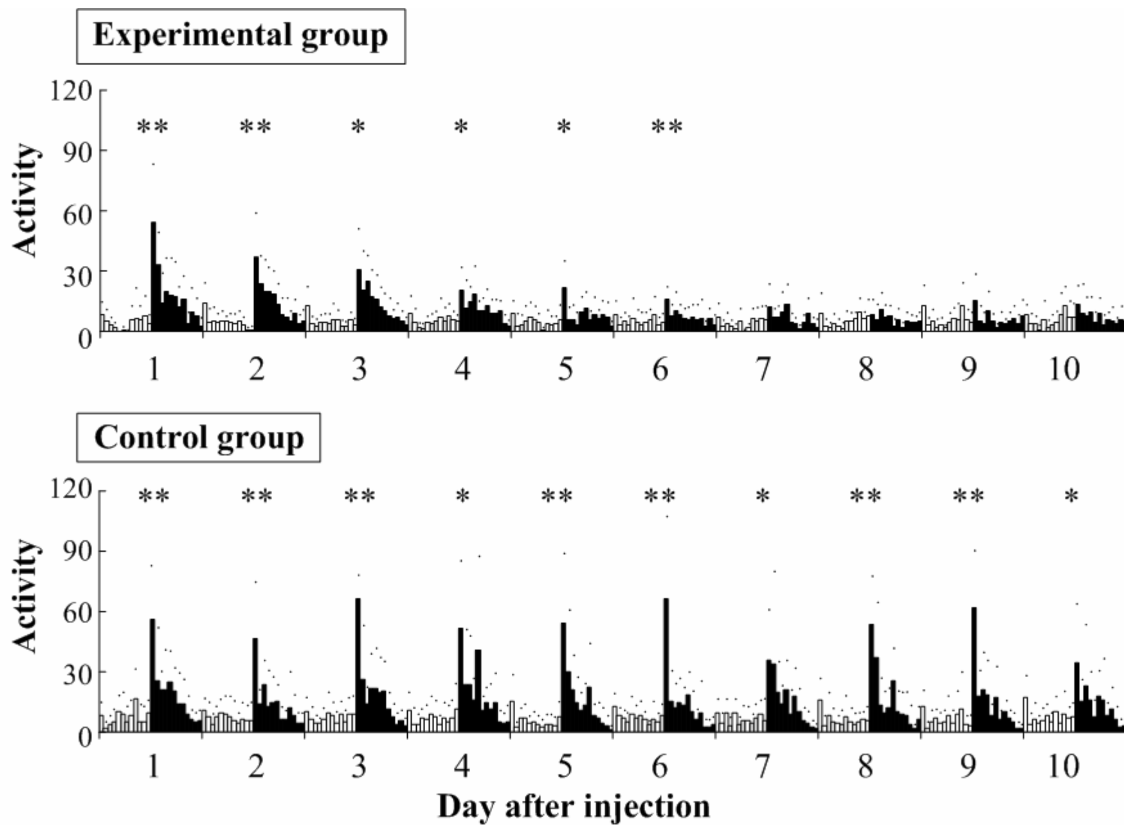

Figure 3-10. The fluctuation of daily locomotion during the first 10 days after treatment under light-dark cycle (L:D=12:12). Each bar in the histogram represents 1 hour of cumulative activities and the dots above each bar indicates the standard error of the mean (n= 11 for the experimental group, n= 13 for the control group). The white bar indicates the day and the black bar indicates the night. Single asterisk (*) denotes average activity of the hour 13 (first hour of the night) different from the average activity of hour 12 (last hour of the day) at the level of $p < 0.05$ (Student's *t*-test). Double asterisks (**): at the level of $p < 0.01$ (Student's *t*-test). Control group: DEPC H_2O injection; Experimental group: *pdf* dsRNA injection.

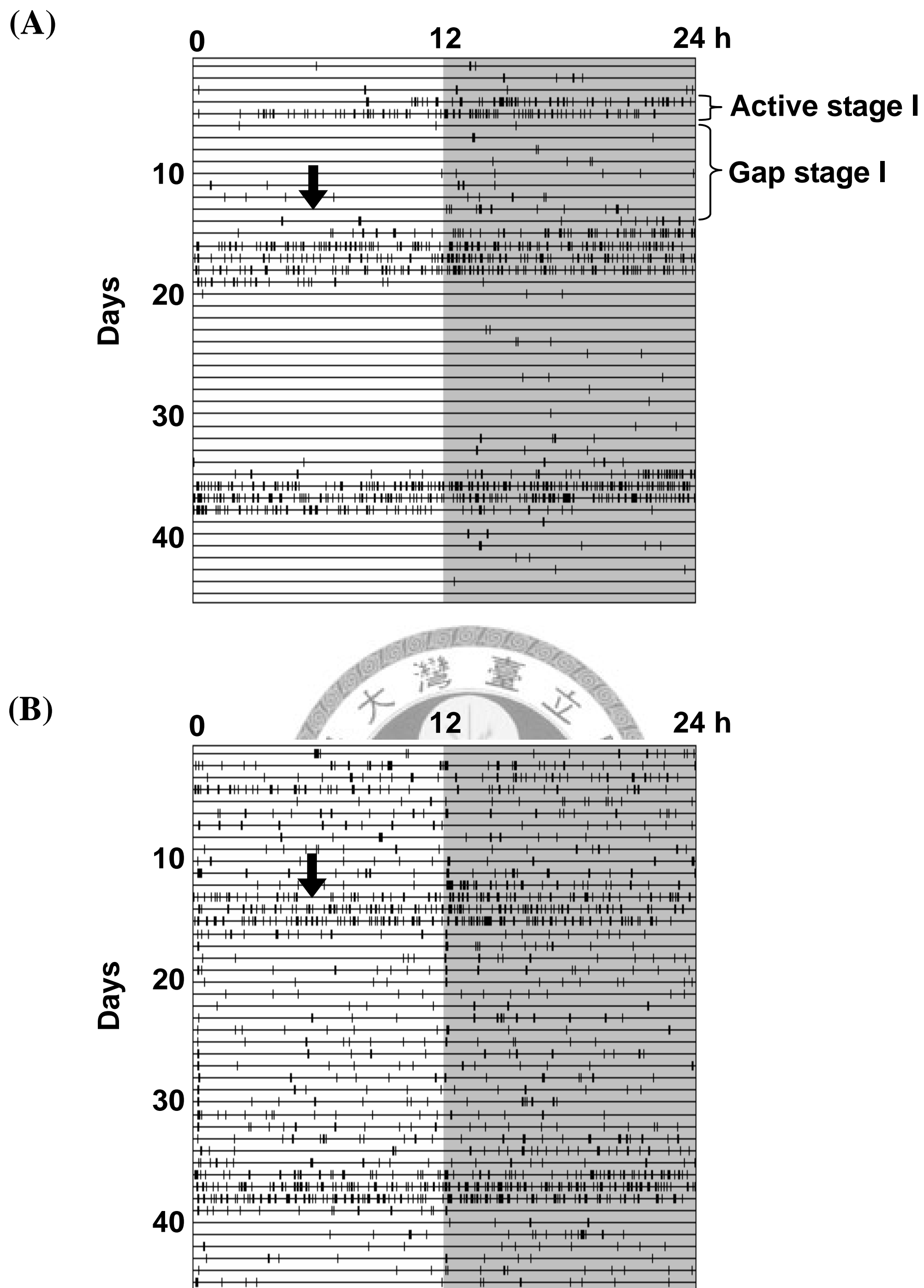

Figure 3-11. Locomotor activity patterns of the female German cockroaches from *pdf* RNAi experimental (A) and control (B) groups under light-dark cycles (LD = 12:12h). The gray background on the actogram indicates the dark condition of the environment, while the white color indicates the light phase. The arrow on the actogram indicates the time point of injection.

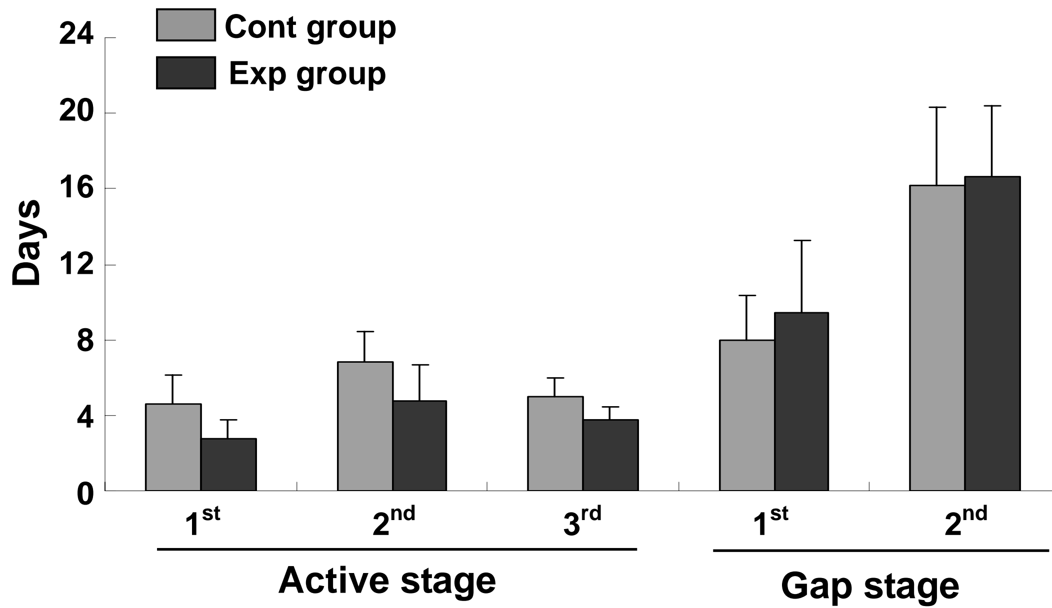

Figure 3-12. The durations of different stages in the actogram of the female cockroach under light-dark cycles (LD = 12:12h). Cont: DEPC H_2O injection; Exp: *pdf* dsRNA injection.

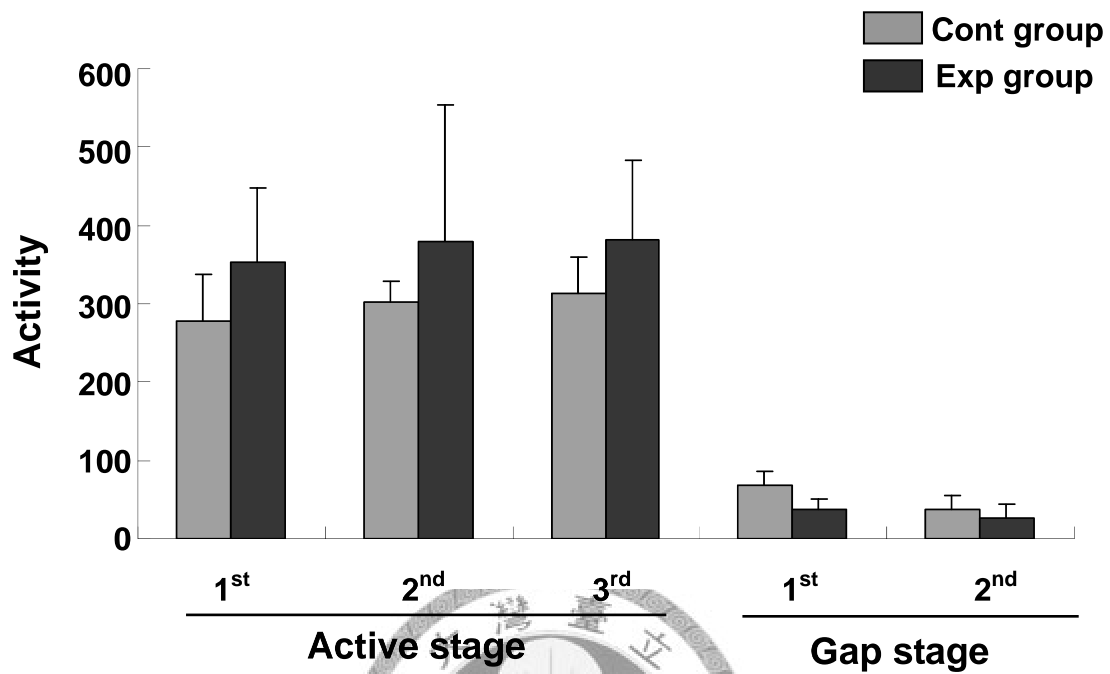

Figure 3-13. The average daily activity of different stages in the actogram of the female cockroach under light-dark cycles (LD = 12:12h). Cont: DEPC H_2O injection; Exp: *pdf* dsRNA injection.

Chapter 4

Conclusions and General Discussion

In this study, I found that two types of the *pdf* mRNAs were transcribed in the German cockroach (Fig. 2-1). The difference between two *pdf* cDNAs was situated in the 5' untranslated region; therefore, only one type of PDF precursor would be synthesized. The sequences of cDNA and PDF of the German cockroach were very similar to *Gryllus bimaculatus, Acheta domesticus*, *Periplaneta americana*, *Romalea microptera*, and *Meimuna opalifera* (Chuman et al., 2002; Rao and Riehm, 1993; Sato et al., 2002; GeneBank: U42472). The PDF precursor of the German cockroach was predicted to be a secretory peptide (Table 2-5), and this was a necessary character for the output factor of the circadian clock.

Transcription of the *pdf* gene of German cockroach was arrhythmic (Fig. 2-6), so its transcription might be not regulated by the clock genes. In the optic lobe, only three groups of cell could express the *pdf* gene (Fig. 3-3, 3-5). One group of cells were located in the proximal frontoventral region, this group of cells were the most possible candidate of the locomotor circadian clock. Whether the translation of *pdf* mRNA was rhythmic or not still remained as an unsolved question, because there were no proper techniques could be used.

The expression of *pdf* gene could be effectively silenced by RNA interference (Fig. 3-1). Without PDF, the locomotion of the male cockroach would become arrhythmic (Fig. 3-6, 3-7). Two possible reasons were responsible for the rhythm losing results. First, the PDF might be the sole output factor of the circadian clock to evoke the activity of the male cockroach. No matter under the light-dark cycle or constant darkness environments, the locomotor activity of the *pdf* dsRNA-injected male cockroaches reduced to a low level (Fig. 3-9). This result indicated that the locomotion did not be started up. If the circadian clock could secrete other molecules to start the locomotion, the locomotor activity decreasing result would not happen. So, this suggests that the

PDF was the only output factor for starting the locomotion during the night. Second, the PDF was the coordinator of the bilateral circadian clocks. Without the coordinator, each circadian clock would tick on its own pace, and the locomotion would be started at different time points within one day, so the locomotor rhythm was finally disturbed. Under light-dark cycle, two circadian clocks could be reset by the light everyday; therefore, the percentage of arrhythmic cockroaches was lower than the percentage of constant darkness group (Table 3-1).

In conclusion, this research proves that PDF is the output factor of the locomotor circadian clock, and PDF plays an important role in the mechanism of circadian rhythm of the German cockroach.

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