國立台灣大學生物資源暨農學院昆蟲學研究所
碩士論文
Graduate Institute of Entomology
College of Bioresource and Agriculture
National Taiwan University
Master Thesis

青春激素誘發德國蜚蠊雌蟲基因表現之分析
Analysis of genes upregulated by juvenile hormone in the
female German cockroach, *Blattella germanica* (L.)
(Dictyoptera: Blattellidae)

陸子鈞
Tzu-Chun, Lu

指導教授：李後晶 博士
Advisor: How-Jing Lee, Ph.D.

中華民國一〇二年八月
August, 2013

## 誌謝

「生命中的遺憾，會引導你去該去的地方」。四年的研究生身分要結束了，比其他同學晚了一倍的時間才完成學業倒不是因為研究多麼費時，而是因為遇到意外。回首過去四年，這些遇到的意外未必稱得上「遺憾」這麼沈重的嘆息，但也確實帶我去到該去的地方。

研究所歲月中，最感謝的是李後晶老師，除了在研究上的指導之外，亦在我的生涯規劃上盡可能給予協助和支持，提供足夠的空間讓我可以探尋自己的興趣還有訂定未來方向。李老師也替研究室帶來許多與國際學者交流的機會，像是邀請學者來訪，或者爭取參加國際研討會的機會，擴展了我對研究領域的認識。

能夠順利完成論文題目還有找到未來方向也要感謝黃佳欣學長，他對知識的渴求還有無窮的好奇心深深感染了我，在研究方法還有科學態度上也非常嚴謹，是影響我甚多的前輩。也感謝萬有引力（或解作命運、神）將育賢帶來研究室，他樂天、不受既有成見限制、敢於挑戰權威，對我是莫大的激勵。還有細心處理行政事務讓大家得以無憂從事研究的黃姊、其實在就讀研究所前就認識又聊得很投緣的黃稑焜學長、在實驗上非常細心又待人很nice而且貓咪跟女兒都很可愛的劉耘學姊、很文藝又在統計方法上給大家帶來許多中肯建議的書綺學姊。很幸運也很高興能在一間溫馨又能彼此學習成長的研究室完成碩士學位。

最後要感謝在背後默默付出、即使不知道他們兒子去台北幹嘛，還是支持他完成學業的我的爸媽。以及研究所歲月下半場陪我完成學業的鄭雅方，我想她會繼續敦促時而懶散的我完成更多未來的挑戰。還有兩隻陪我念書到深夜，隔天一早又準時用力嘶吼叫我起床放飯的毛小鬼（*Felis silvestris catus*）阿喲和噗啾。

這篇論文所揭示的發現，未必在科學史上具有重大貢獻，但過程中我對自己還有所處的外在環境的認識，卻對我意義非凡。四年過去，在這篇論文付梓之際，期許自己未來仍保有對世界萬物的好奇心。

「你以恩典為年歲的冠冕，你的路徑都滴下脂油。」—詩篇 65：11

# 中文摘要

德國蜚蠊（*Blattella germanica*）雌蟲的發育、生殖及行為受到體內青春激素濃度的調控。本研究即利用青春激素類似物－烯蟲酯（methoprene）施用於三日齡的雌成蟲，並藉由抑制消除雜合技術（Suppression Subtractive Hybridization, SSH），找出施用烯蟲酯後特殊表現的基因。雜合後的cDNA資料庫，包含20個特殊表現的基因片段，這些基因潛在受到青春激素濃度調控。與線上資料庫比對的結果，其中9個基因片段，其中功能各涉及卵巢發育、能量補給、蛋白產物合成、細胞分裂、解毒等，另外有11個基因則功能未知。功能已知的16號基因，和能量調控有關，但尚未有更詳細的研究；再加上三個功能未知的基因，共四個基因，由進一步研究得到更多資訊。藉由即時聚合酶連鎖反應（real-time PCR）技術，確認這四個基因受到青春激素誘發表現，也顯示在不同日齡的雌蟲間有不同的表現量；此外，在不同器官也有不同表現量，暗示著可能各有不同功能。由於過去研究發現，青春激素濃度會間接調控德國蜚蠊雌蟲的日週律動以及卵巢發育，因此我們也藉由RNAi技術，測試這四個基因是否屬於調控日週律動或卵巢發育的調控因子。結果顯示，注射雙股RNA後，四個基因的表現量降低，其中三個基因BGME02、BGME05及BGME16的表現量被抑制之後，卵巢的發育減緩，但日週律動卻無明顯差異。因此我們認為這三個基因是青春激素下游調控卵巢發育的基因，但不直接調控雌蟲的日週律動。
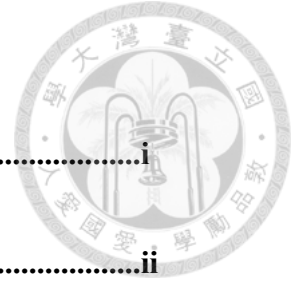

關鍵詞：德國蜚蠊、青春激素、日週律動、RNAi、抑制消除雜合法

# Abstract

Juvenile hormone (JH) is the major hormone controlling the development, behavior and reproductive cycles of the female German cockroach, *Blattella germanica* (L.) (Dictyoptera: Blattellidae). In this study, JH analog - methoprene was topically applied on 3-day old females before with Suppression Subtractive Hybridization (SSH) to identify the genes that were particularly expressed. The SSH cDNA library composed with 20 distinctive sequences of genes. Nine of those genes were involved in ovarial development, energy supply, protein synthesis, cell division or detoxification, and rest of genes possessed unknown functions. For functional studies, RNAi was used to knock-down the expression level of four genes, and dsBGME02, dsBGME05 and dsBGME16 injection inhibit ovary development, but RNAi of all four genes do not affect the locomotor rhythmicity. Based on these findings, we found three genes, which are BGME02, BGME05 and BGME16 mediated by JH downstream regulation and ovary development, but do not participated in control of locomotion.

**Keywords**: *Blattella germanica*, Circadian rhythm, Juvenile Hormone (JH), RNAi, Suppression Subtractive Hybridization

# Contents

# List of Tables

# List of Figures

# Introduction

Juvenile hormone (JH) is one of the major hormones in adult female German cockroach, *Blattella germanica* (L.) (Dictyoptera: Blattellidae), controlling the development (Gadot et al., 1989), locomotor behavior (Lin and Lee, 1998) and reproductive cycle (Belles et al., 1987). The JH level shows a cyclic pattern during reproductive cycle, it rapidly increases after emergence and suddenly decreases after formation of ootheca, and is remaining at low level until next gonadotrophic cycle (Chiang and Schal, 1994; Lin and Lee, 1998, Treiblmayr et al., 2006).

The production of JH by corpora allata (CA) is regulated by both internal and environmental stimuli (reviewed by Schal, 1997), such as mating status (Gadot et al. , 1989) and food quality (Cooper and Schal, 1992a, 1992b). Suazo et al. (2009) used RNAi to silence the Bla g 1, digestion-related gene (Gore and Schal, 2004; 2005), the ovary development was inhibited. However, it can be rescued by applying JH. The results sugested that JH synthesis was regulated by nutrient condition. Moreover, by knock-down the expression of BgTOR gene, Maestro et al. (2008) found that the "target of rapamycin" (TOR) pathway mediated nutritional signal and JH synthesis in corpora allata of German cockroach. Furthermore, the starvation inhibited the Forkhead-box O (FoxO) transcription factor, which inhibited JH synthesis (Süren-Castillo et al., 2012).
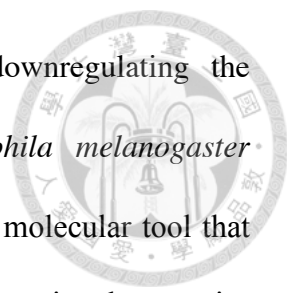
In female adult German cockroach, JH regulates not only the reproductive physiology, vitellogenesis and oocytes development (Tobe and Stay, 1985; Scharrer, 1987; Gadot et al., 1991), but also behaviors such as sexual receptivity and locomotor activity (Liang and Schal, 1994; Schal and Chiang, 1995; Lin and Lee, 1996; Lin and Lee, 1998). When virgin female adults are sexually mature, their locomotor activities

become much higher and show arrhythmic patterns (Lin and Lee, 1996). Even though the allatectomized females do not show high locomotor activities, their locomotor activities can be rescued by JH analog apply (Lin and Lee, 1998).
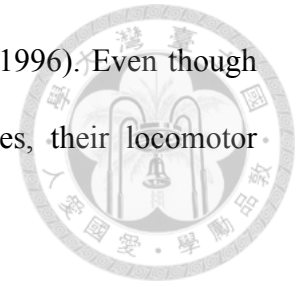
Suppressing Subtractive Hybridization (SSH) is a technic that can identify the differences in relative abundance of cDNA between two different cDNA samples (Diatchenko et al., 1996). In insects, SSH has been shown to be effective to identify the genes that differently express between two groups, such as the diapause-specific gene in the northern house mosquito (Culex pipiens) (Robich et al., 2007), the immune-related genes in kissing bug (*Rhodnius prolixus*) (Ursic-Bedoya and Lowenberger, 2007), greater wax moth (*Galleria mellonella*) (Seitz et al., 2003), tobacco hornworm (*Manduca sexta*) (Zhu et al., 2003), red flour beetle (*Tribolium castaneum*) (Altincicek et al., 2008), burying beetle (*Nicrophorus vespilloides*) (Vogel et al., 2011), locust (Zhang and Xia, 2009). In German cockroach, SSH was used to identify the genes related to choriogenesis (Irles et al., 2009), compare the difference of gene expression between wild-type and resistant strain (Pridgeon et al., 2003; Pridgeon and Liu, 2003). In this study, SSH will be used to identify the genes that upregulated by juvenile hormone. Since the sexual maturation is synchronized with the fluctuation of JH titer (Chiang and Schal, 1994; Lin and Lee, 1998), in order to eliminate the age effect on JH pathways, methoprene (JH analog) was applied on 3-day-old female adults to mimic the high level of JH (Zhu et al., 2009).

RNA interference (RNAi) is a molecular tool that can silence the complementary endogenous messenger RNA (mRNA) by appling exogenous double-stranded RNA (dsRNA). After RNAi was discovery in the nematode *Caenorhabditis elegans* (Fire et

al., 1998), the first successful RNAi experiment in insects is that downregulating the *frizzled* and *frizzled 2* genes by dsRNA injection in *Drosophila melanogaster* (Kennerdell and Carthew, 1998), and it was rapidly developed as a molecular tool that widely applied in variety orders of insecta and used to study the functional genomics and pest control (Huvenne and Smagghe, 2010; Burand and Hunter, 2013; Belles, 2010), such as Diptera (Lum et al., 2003; Dietzl et al., 2007), Lepidoptera (Yu et al., 2008; Terenius et al., 2011), Hymenoptera (Schluns & Crozier, 2007; Antonio et al., 2008), Coleoptera (Arakane et al., 2004; Suzuki et al., 2008), Orthoptera (Dong and Friedrich, 2005), Hemiptera (Xu and Han, 2008), Isoptera (Zhou et al., 2008) and Blattodea, especially in *Blattella germanica* (Ciudad et al., 2006; Maestro and Belles, 2006; Maestro et al., 2008; Irles et al., 2009; Lee et al., 2009; Suazo et al., 2009; Huang and Lee, 2011; Süren-Castillo et al., 2012). There are three main methods of dsRNA delivery to insects: injection, feeding and soaking, although feeding and soaking are cheaper and easier than injection, dsRNA can immediately into the hemolymph by injection and thus avoiding possible barriers, like gut epithelium or cuticle in feeding or soaking approach, on the other hand, researches can know the exact amount of dsRNA that injected, therefore, injection is still widely used for dsRNA delivery (Yu et al., 2013).

Although there are many studies investigating the physiology that regulated by JH, JH regulatory pathway at molecular level is largely unknown. It is our intention to use SSH to identify some genes that upregulated by JH in this study. Since the JH regulates the reproduction and locomotor activity in female German cockroaches, we expected that we can obtain some genes that related with ovary development, energy metabolism through SSH. In order to speculate the possible function of target genes that

3

obtain through SSH, we knockdown the expression level of target genes by RNAi then measure the length of ovary and daily locomotor activities, if the target genes related with ovary development or locomotor activity, then the RNAi would disrupt the normal condition of those two phenomenons.
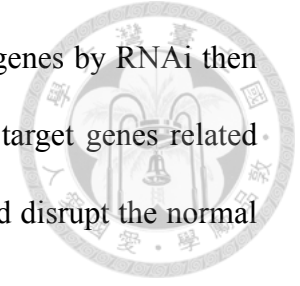
# Materials and Methods

## Insect culture
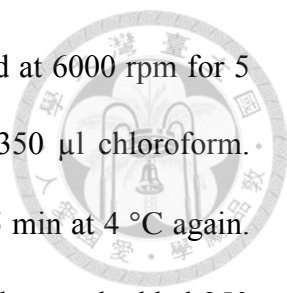
The German cockroach, *Blattella germanica* (L.) were reared with water and dog chow in an environmental chamber under 28°C, and light-dark cycle of 16:8 h condition. Newly emerged adults were separated by sex.

## Methoprene application

The reproductive cycle in German cockroach is regulated by JH. To mimic the effect of JH, 1mg of methoprene (JH analog) was dissolved in 200μl acetone as stock, and topically 20μl of stock applied on 3-day-old female adults (Comas, 1999) as the treated group, the control group are topically applied with acetone only. In order to eliminate the daily fluctuation of gene expression, all samples were collected at Zeitgeber Time (ZT) 7-8 constantly.

## Total RNA extraction

The total RNA samples were collected at 4 time points: ZT 3, 6, 18 and 24 after the methoprene topical application. Six individuals were collected at each time point for each group. The wings and legs of cockroaches were removed before putting in 1.5 ml microcentrifuge tubes with 400 μl TRIzol® reagent (Invetrogen). The microcentrifuge tubes with samples were frozen in liquid nitrogen before homogenized. The samples were homogenized at the time when half of the reagent in the tube melted by using an electronic stirring on ice. Female German cockroach contained abundant of lipid and fat that can interfere with RNA extraction. Before extracting RNA the following pre-treatment was needed to remove most of fat from the sample: Added 400
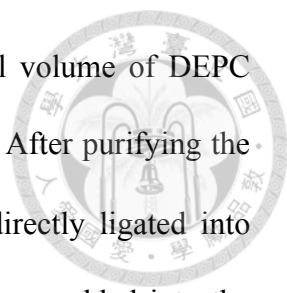
μl chloroform and mixed well by vortex for 15 sec, then centrifuged at 6000 rpm for 5 min at 4 °C. Removed 350 μl from the lower layer, then added 350 μl chloroform. Mixed well by vortex for 15 sec, then centrifuged at 6000 rpm for 5 min at 4 °C again. Transferred 250 μl of the upper layer into a new microcentrifuge tube, and added 250 μl TRIzol® and extracted RNA according to the TRIzol® official protocol twice. 2 μg total RNA as template to synthesize cDNA by High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems).

**Gene expression comparison and cDNA library construction.**

SSH was performed with the PCR-selected cDNA Subtraction Kit (Clontech) by following the manufacturer's protocols. The tester library was methoprene-applied group and driver library was acetone-applied group. After completed the SSH, the gene sequences were ligated with plasmid vectors and transformed into bacteria. 283 strains of tester and 28 strains of driver library were picked and sequenced. The results of sequencing were clustered by CAP3 program, after then using BLASTx to search the database for homologous gene sequences.

**Gene Cloning**

The PCR products (50 μl) amplified by Taq DNA Polymerase (Super-Run Tag DNA Polymerase, PROTECH) with extra A-base at 3'-terminal were mixed with one-tenth of the sample volume of 3 M sodium acetate and 5 times of the sample volume of 99.8% ethanol. Incubated at -20 °C at least 2 h, and then centrifuged at 19000 g for 39 min at 4 °C. The supernatant was discarded. The nucleic acid pellet was washed with 70% ethanol, and then centrifuged at 19000 g for 10 min at 4 °C. Repeated the washing steps again, then, discard the supernatant and dried the sample by a vacuum

6
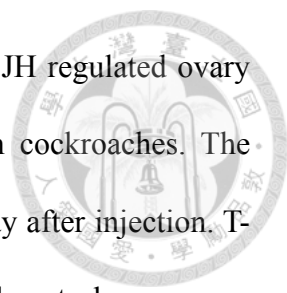
concentrator. Finally, dissolved the nucleic acid by adding optimal volume of DEPC (diethyl pyrocarbonate)-treated $H_2O$, and heated at 55 °C for 5 min. After purifying the nucleic acid sample with ethanol precipitation, the product was directly ligated into pGEM®-T Easy Vector (Promega). One of 10 µl ligation product was added into the competent cell (ECOSTM, Yeastern Biotech), and heat-shocked it for transformation. The transformed cells were added into l ml LB (Lysogeny Broth) with 1 mg Ampicillin for at least 2 h before spreading them on medium. Medium with cloning bacteria was incubated at 37 °C for 8-14 hours.

**RNA interference**

The double-strand RNA of BGME02, BGME05, BGME07, and BGME16 was synthesized by MEGAscript® Kit (Applied Biosystems) following the manufacturer's protocol. The double-strand (ds) RNA was diluted with DEPC-treated water into 3 µg/µl. At ZT 7-8, 1µl dsRNA was injected into the cockroach between third and fourth abdominal sternites. Two control groups were injected with dsEGFP (exogenous enhanced green fluorescence protein) and DEPC-treated water (sham) respectively.

In order to identify the four genes that were involving in the JH regulated locomotion, dsRNA was injected into 1-day-old female German cockroaches, then recorded the locomotor activity by TriKinetics System individually for 8 days. The cockroaches were kept separately in a glass tube (16 cm long, 2 cm wide) with food and water. The locomotion monitoring system was kept in an environmental chamber under 28 °C and LD: 16:8 h conditions. Chi-sequare peridogram was use to analyze the rhythmicity of daily locomotion (Sokolove and Bushell, 1978).

In order to identify the four genes that were involving in the JH regulated ovary development, dsRNA was injected into 1-day-old female German cockroaches. The length of basal oocytes of each ovariole was measured at 1- or 5- day after injection. T-test was used to compare the basal oocyte length between treated and control group.

**Quantification of mRNA**

The efficiency of each primer pairs for qRT-PCR was validated by establishing a standard curve with five serial dilutions. Triplicated PCR reactions were carried out in an ABI StepOne system (version 2.1), using SYBR® FAST qPCR Kit (KAPA). All sets of quantification include both non-template control reactions and a standard sample from cloned plasmid for normalization of data. The PCR program began with single cycle at 95 °C for 10 minutes, 40 cycles at 95 °C for 15 seconds and 60 °C for 60 seconds. Afterward, the PCR products were heated to 95 °C for 15 seconds, cooled down to 60 °C for 15 seconds and heated to 95 °C for 15 seconds, in order to measure the dissociation curves and to determine an unique PCR product for each gene. Expression levels of each gene were calculated relative to the *Blattella germanica* actin expression level using ABI StepOne system.

# Results
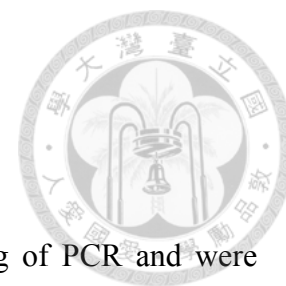
## Suppression Subtractive Hybridization

There were total 283 colonies that confirmed by sequencing of PCR and were clustered by CAP3 program into 20 contigs. After comparing the sequences with NCBI database by BLASTx, the cDNA library was constructed (Table 1). Among the 20 sequences, 7 sequences were no homology to database and 4 sequences had no known function. The 3 sequences (BGME02, BGME05, BGME07) in the cDNA library without homology and can be picked primer by Primer 3 program were chosen for functional study. In addition, BGME16 was also chosen to clarify its putative function in protein and carbohydrate metabolism which was found in *Bombus terrestris* (Table 1). Since virgin females were highly active, which was synchronized with the increase titer of JH, BGME16 might be involved in energy supply for locomotion or reproduction. Totally, the 4 genes were chosen as targets for functional study by RNAi and qRT-PCR.

Table 1. The cDNA library of 20 partial sequences obtained via SSH in female German cockroaches after acceleration of sexual maturation by topical application of methoprene (juvenile hormone analog).

| cluster ID | Length (BP) | Best BLASTx | E-value of BLASTx | Hit_accession |
|---|---|---|---|---|
| BGME01_1 | 64 | No homology | - | - |
| BGME01_2 | 237 | unknown [*Picea sitchensis*] | 8.00E-13 | ABK26259.1 |
| BGME01_3 | 205 | hypothetical protein [*Opisthacanthus cayaporum*] | 5.00E-06 | CAX51406.1 |
| BGME01_4 | 897 | cytochrome oxidase subunit I [*Microcerotermes sp.*] | 2.00E-25 | AEI25867.1 |
| BGME02* | 344 | No homology | - | - |

| | | | | |
|---|---|---|---|---|
| BGME03 | 344 | vitellogenin [*Blattella germanica*] | 7.00E-70 | CAA06379.2 |
| BGME04 | 325 | 60S ribosomal protein L18a [*Triatoma infestans*] | 9.00E-44 | ABR27953.1 |
| BGME05* | 468 | No homology | - | - |
| BGME07* | 335 | No homology | - | - |
| BGME08 | 293 | vitellogenin [*Blattella germanica*] | 5.00E-06 | CAA06379.2 |
| BGME09 | 581 | fibroblast growth factor 9 | 2.00E-53 | EDM14369.1 |
| BGME10 | 85 | No homology | - | - |
| BGME11 | 103 | similar to flightin CG7445-PA [*Tribolium castaneum*] | 4.00E-17 | XP_969293 |
| BGME13 | 74 | hypothetical protein [*Blattella germanica*] | 1.00E-06 | CAR94552 |
| BGME14 | 618 | cytochrome b | 1.08E-79 | YP_003002162 |
| BGME15 | 710 | No homology | - | - |
| BGME16* | 395 | Putative glycerol kinase 3 [*Bombus terrestris*] | 1.00E-170 | XP_003393109 |
| BGME17 | 190 | No homology | - | - |
| BGME18 | 148 | Peptidyl-prolyl cis-trans isomerase [*Blattella germanica*] | 4.00E-101 | P54985.1 |
| BGME19 | 206 | Parcxpwnx03 (function-unknwon) [*Periplaneta americana*] | 3.00E-18 | AAW57774 |

* For further studies.


## Methoprene application induce the expression of 4 target genes

The expression level of the four target genes were induced significantly by adding methoprene on 3-day-old females (Fig. 1). The qRT-PCR results suggested these 4 genes were not false positive result in SSH (T-test $p=$ 0.05, 0.011, 0.02, 0.003 for BGME02, 05, 07, and 16 respectively).
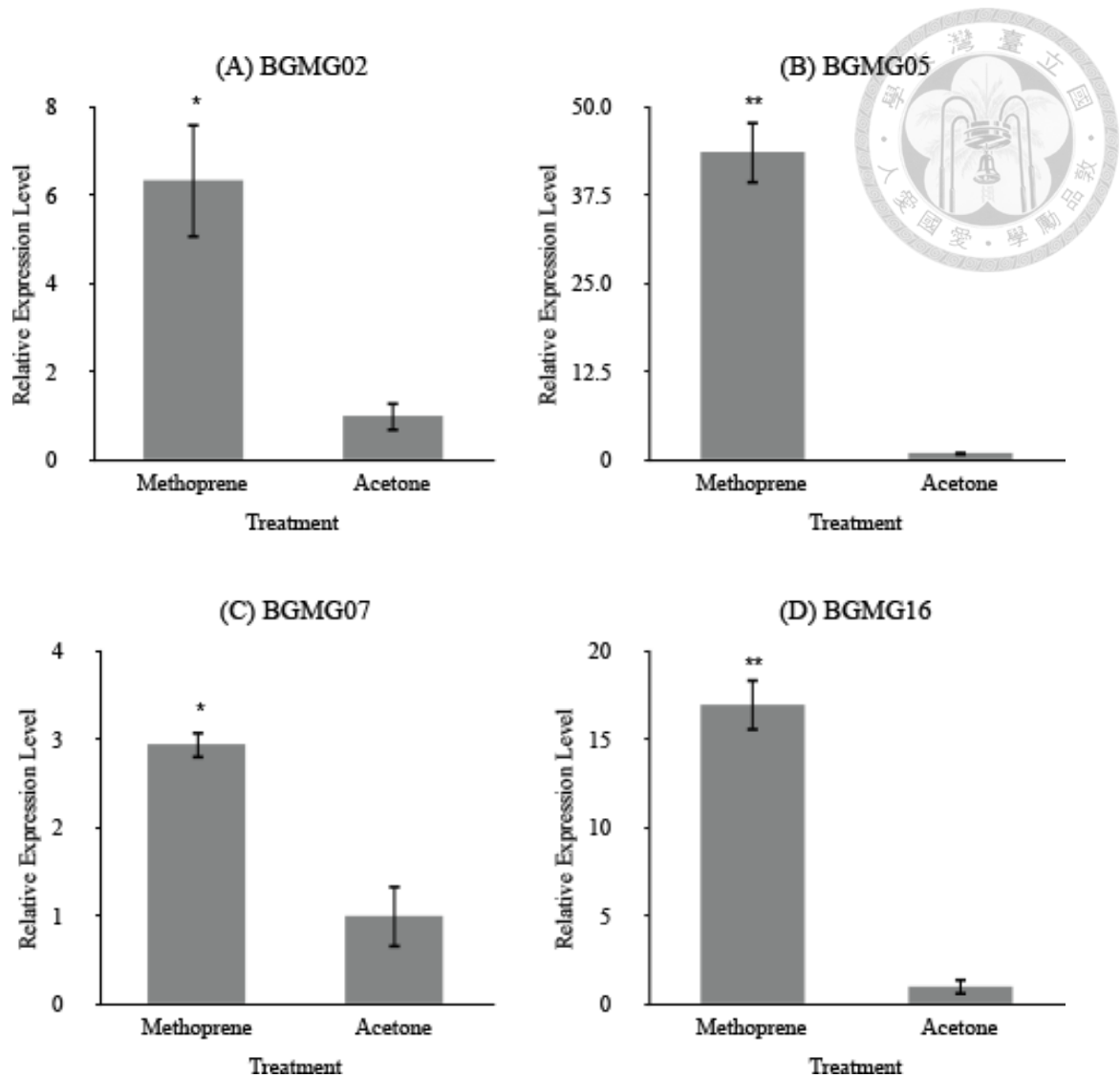
Fig. 1. The expression pattern of 4 novel genes: (A) BGME02, (B) BGME05, (C) BGME07, (D) BGME16 1 day after methoprene topical application on virgin female German cockroaches (t-test, * $p<0.05$, ** $p<0.01$ N=3).

## Daily fluctuation of expression level of 4 target genes

The daily fluctuation of expression level of the 4 target genes in the first reproductive cycle were shown in Fig. 2. The relative expression level that compared with day 1 increased significantly in the first part of reproductive cycle and peaked at day 7. Then, it dropped significantly afterword. The fluctuation pattern was coincided with pattern of locomotor activity, the ovary development and JH titer.
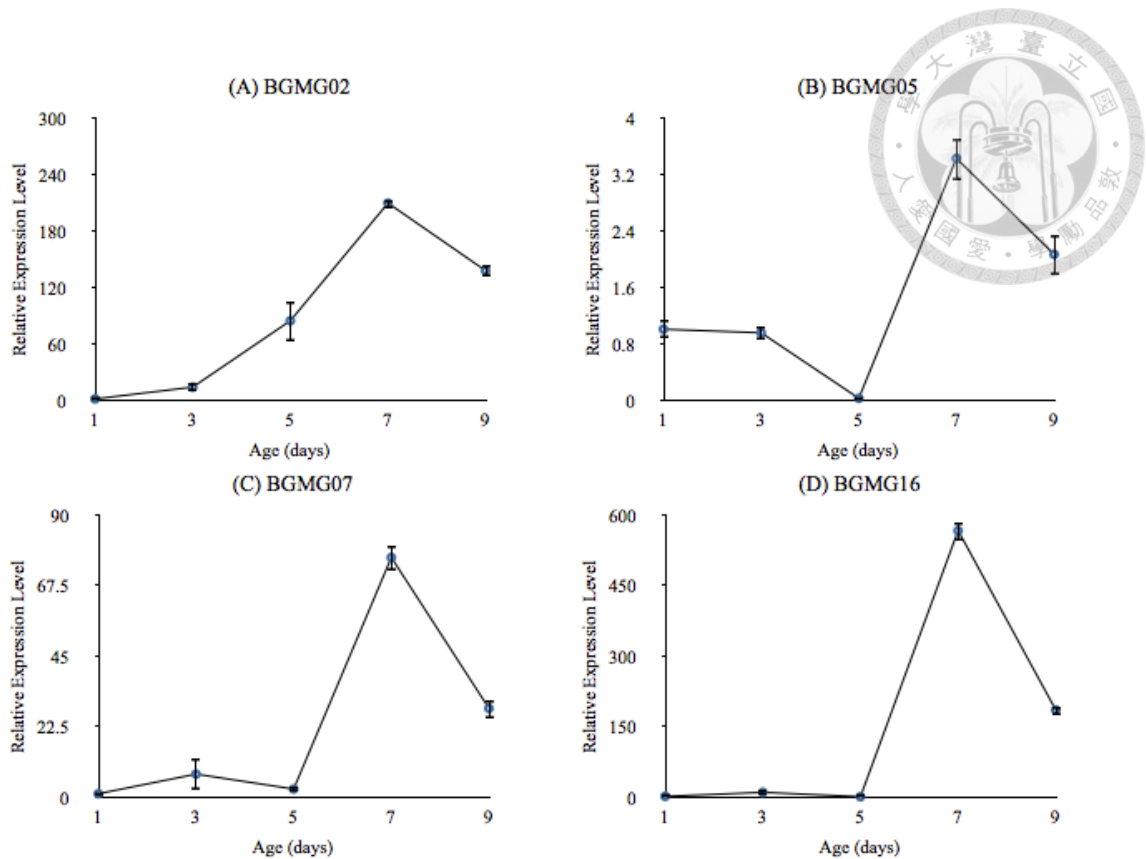
Fig. 2. Daily fluctuation of expression level of 4 novel genes: (A) BGME02, (B) BGME05, (C) BGME07, (D) BGME16 during the first reproductive cycle of virgin female German cockroaches. (N=3)

**Expression level of the target genes in internal organs**

The gene expression levels of mRNA in 6 different organs (ventral nerve cord, fat body, head, legs, midgut and ovary) of 3-day-old female German cockroaches were shown in Fig.3. Although a few genes expressed nearly undetectable in some organs (like BGME07 in ovary and BGME16 in legs), the reference gene (actin) was detected in all organs. It indicated that the PCR results were reliable. All of the four genes showed different expression levels among organs (ANOVA $p<0.01$ and followed by HSD post hoc test).

The differential expression of the target genes among various internal organs indicated potential functions of the genes. Higher expression of BGME02, BGME05 and BGME16 in fat body than other organs implied its possible function in protein and

carbohydrate metabolism, energy supply, reproduction or detoxification which were related the physiological functions of fat body. However, the possible function of BGME05 and 16 in reproduction would become minimum because of less expression in ovary. The high expression level of BGME02 in midgut might imply its involvement in regulation of nutrient and energy intake which might lead to protein synthesis and energy supply from fat body for development of ovary.
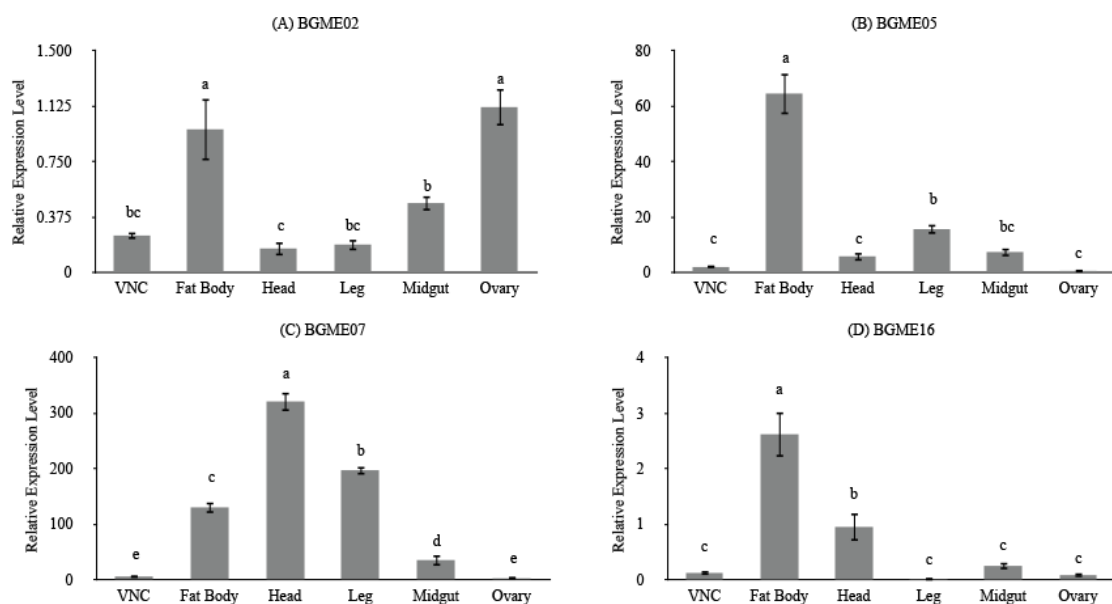


Fig. 3. The expression level of four novel genes (A) BGME02 (B) BGME05 (C) BGME07 (D) BGME16 in various internal organs of 3-day-old virgin females. The relative expression level was quantified by qRT-PCR and compared with internal standard. VNC: ventral nerve cord. Different letters on the chart indicate significant differences (ANOVA, $P<0.05$; HSD for post hoc test, N=3).

**RNAi reduce gene expression levels**

The RNA interference method has been demonstrated to be effective in knocking down the 4 target genes (Fig. 4). The injection of double strand RNA significantly reduced the expression level of the 4 genes. This silencing effect could last for at least 5 days (Fig. 4B).

(A)
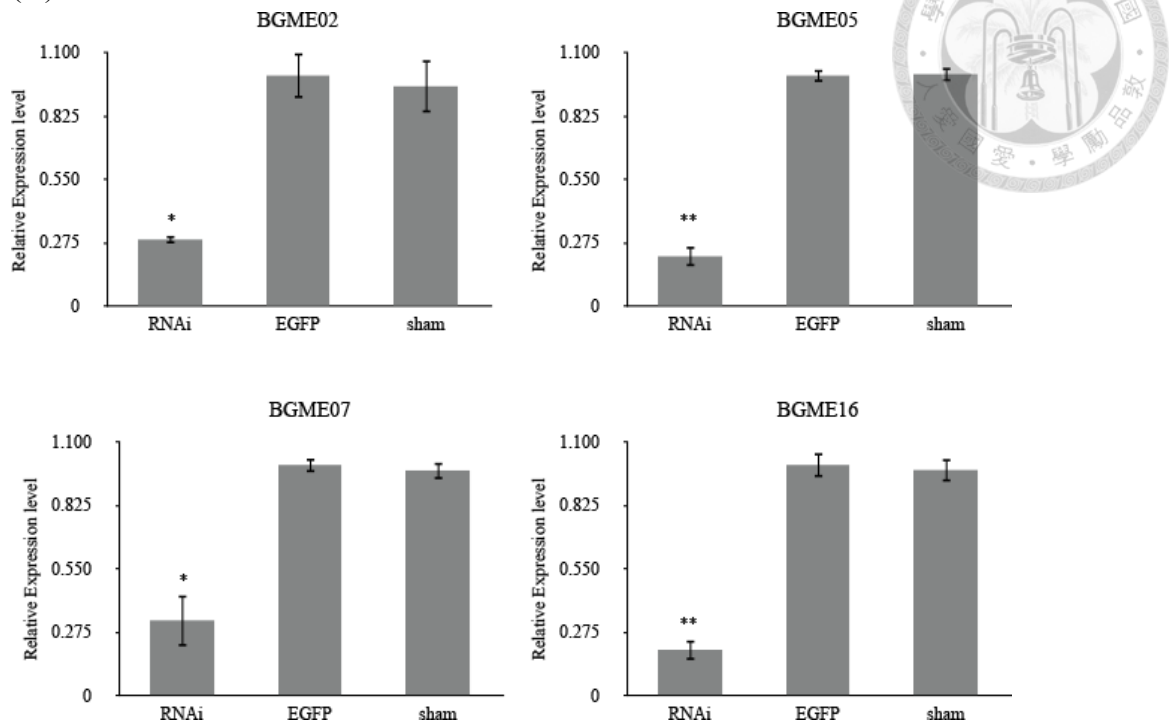


(B)
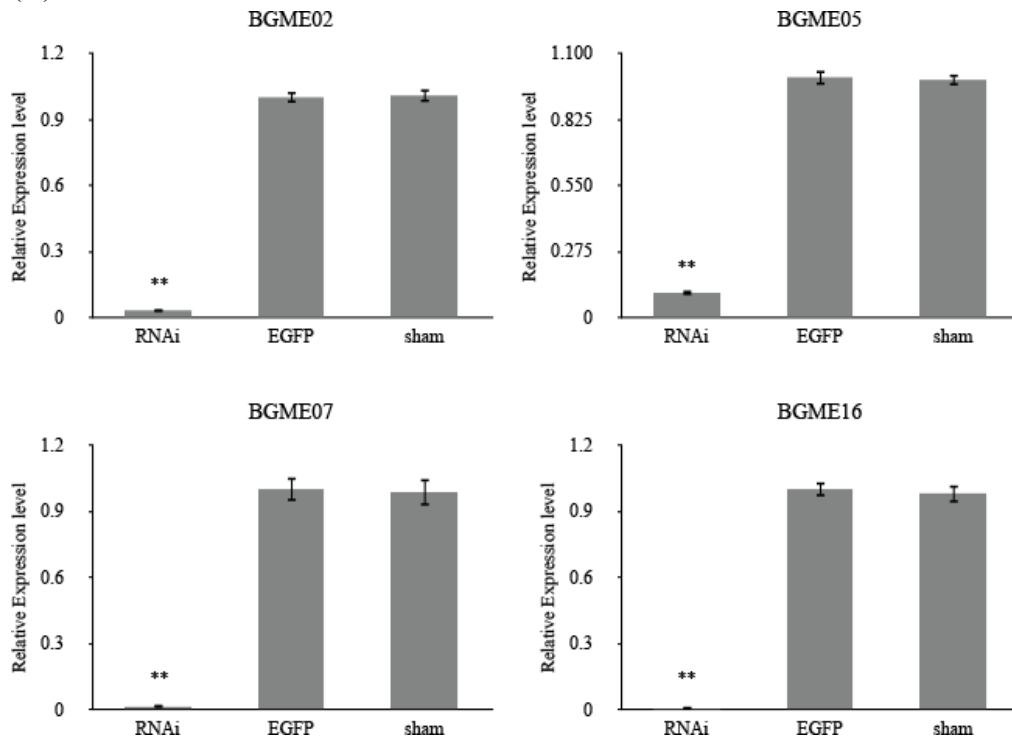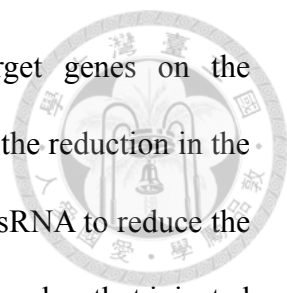


Fig 4. The gene expression levels of virgin female adult of the German cockroach (A) one and (B) five days after dsRNA injection on 3-day-old cockroaches. (N=6 for RNAi, N=3 for EGFP and sham, t-test $p<0.01$)

**RNAi inhibit the Development of Ovary**

The knockdown effect of injecting dsRNA of the 4 target genes on the development of ovary was shown in Fig. 5. The RNAi did not cause the reduction in the length of oocyte after 2 day of dsRNA injection. It look 5 days for dsRNA to reduce the growth of oocyte. The basal oocyte length of 7-day-old female cockroaches that injected with dsBGME02, dsBGME05 and dsBGME16 were significant shorter than control group. However, dsBGME07 injection did not affect the basal oocyte length (Fig. 5B). These result indicated BGME02, 05 and 16 were involved in ovary development, but BGME07 was not.
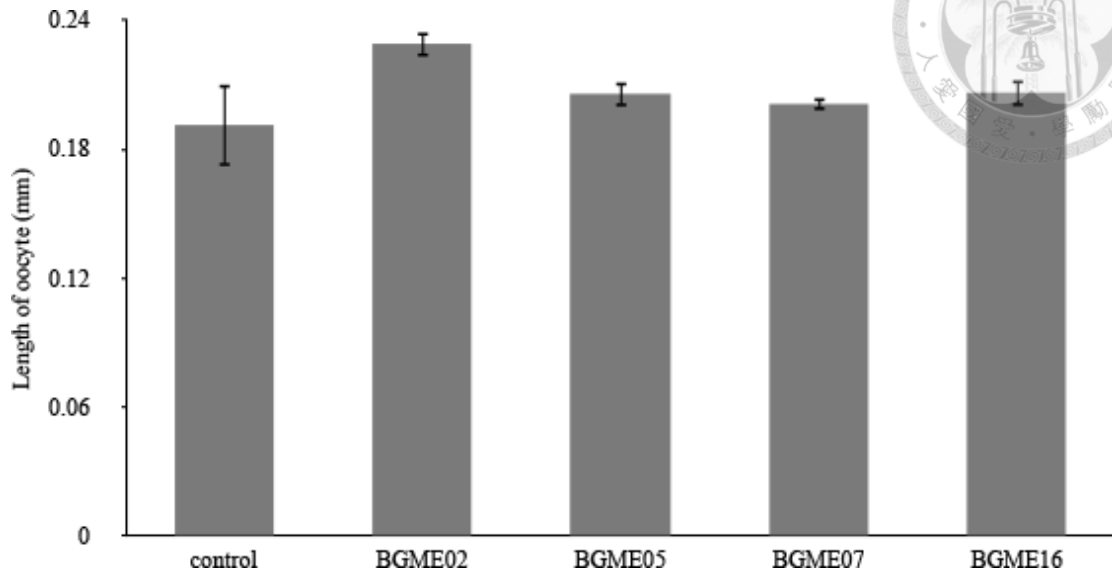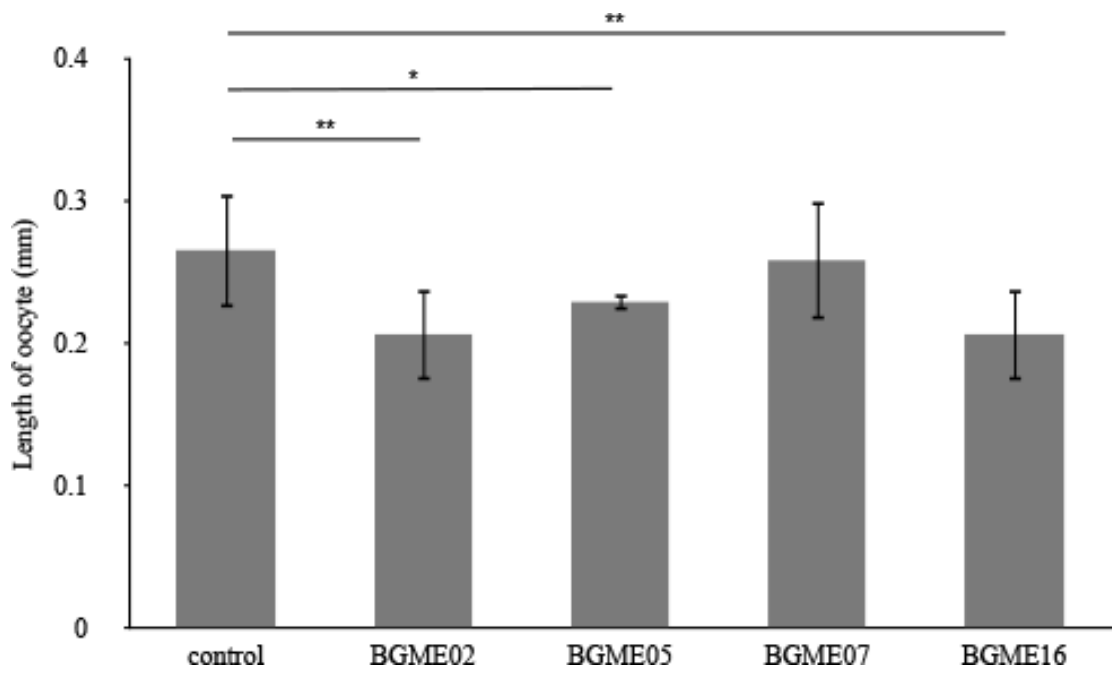
Fig 5. The silencing effect of the 4 target genes on ovary development of the German cockroach after 2 days (A) or 5 days (B) injection of dsRNA on 1-day-old virgin females. (t-test *p*<0.05)

**RNAi affect the locomotor activity**
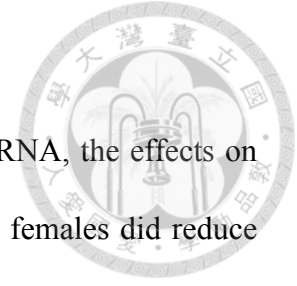
When the 4 target genes were knocked down by injecting dsRNA, the effects on locomotion were shown in Table 2. Even though the RNAi treated females did reduce the percentage of non-rhythmic locomotion from 75% to 41%, the statistic analysis did not show significant difference in reduction. This finding indicated the non-involvement of the 4 genes on circadian locomotion. The non-rhythmic locomotion was also demonstrated with the data of temporal distribution of locomotion. In addition, the total amount of locomotion remained the same after RNAi treatment. These results indicates the 4 genes did not involve in the regulation of locomotion and circadian clock.

Table 2. The effects of injecting dsRNA of the 4 target genes on locomotion of virgin femal cockroach. (t-test P>0.05)

| Treatments | non-rhythmic locomotion (%) | N | Temperal distribution of locomotion (%) | | | Total activity (mean ±S.E.) |
|---|---|---|---|---|---|---|
| | | | Day 5 | Day 6 | Day 7 | |
| control | 75 | 12 | 62.0±4.66 | 62.70±4.34 | 59.26±5.3 | 1900.2±520.5 |
| | | | - | - | - | - |
| dsBGME 02 | 41.67 ns | 12 | 70.41±5 | 68.07±4.38 | 66.40±4.37 | 1822.1±380.1 |
| | | | P=0.23 | P=0.39 | P=0.31 | P=0.9 |
| dsBGME 05 | 54.55 ns | 11 | 70.57±5.13 | 69.0±4.48 | 66.41±4.46 | 2282.1±461.1 |
| | | | P=0.24 | P=0.33 | P=0.32 | P=0.59 |
| dsBGME 07 | 50 ns | 12 | 69.22 ±5.29 | 65.78 ±4.72 | 65.93 ±4.59 | 1635.6±360.9 |
| | | | P=0.32 | P=0.63 | P=0.35 | P=0.68 |
| dsBGME 16 | 80 ns | 10 | 64.79 ±4.5 | 62.62 ±4.49 | 60.61 ±3.98 | 2278±530.2 |
| | | | P=0.67 | P=0.99 | P=0.84 | P=0.61 |

ns: not significant difference, Chi-squar p>0.05

# Discussion

During oogenesis of the German cockroaches, JH from CA is released to trigger the calling behavior (Lee and Wu, 1994; Lin and Lee, 1996) and ovary development. Since the German cockroach posses cyclic reproduction, JH regulation shows cyclic pattern to meet the reproduction. It is assumed that some genes related with energy supply and protein synthesis should be expressed in this period. We obtain 20 partial sequences of genes that were highly expressed after simulated the high concentration of JH (Table 1). They are candidate genes to elucidate the molecular mechanisms of JH downstream regulation.

Two of the twenty sequences (BGME03 and BGME08) belong to vitellogenin gene which can be translated into precursors of vitellins (the major egg yolk protein in insect) (Sappington and Raikhel, 1998). The function of vitellin was confirmed its relationship with ovary development (Comas et al., 1999), and the complete sequences has been fully cloned in the Geman cockroach (Comas et al., 2000). Furthermore, the BGME09 is fibroblast growth factor 9, which involved in the regulation of embryonic development, cell proliferation and cell differentiation (Galzie et al., 1997). Besides, two genes are related with protein synthesis: 60S ribosomal protein L18a (BGME04) that encodes a ribosomal protein of the 60S subunit (Adams et al., 1992), and peptidyl-prolyl cis-trans isomerase (BGME18) that participated in the process of protein folding and trafficking (Martinez and Hegardt. 1995). Moreover, one of those genes is cytochrome b (BGME14), a component of respiratory chain complex III (Esposti et al., 1993), which related with ATP synthesis and energy supply. All together, those function-known genes meet our assumption that related with energy supply and protein synthesis, except the COXI, a detoxify gene that higher expression in resistant pyrethroid resistant

strain of the German cockroaches (Pridgeon and Liu, 2003). The detoxify gene (BGME01_4) probably was induced by reacting to exogenous chemical (methoprene) rather than up-regulated by JH.
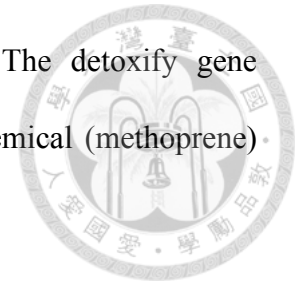
Three of eleven function-unknown genes (BGME02, BGME05, BGME07) which sequences are long enough to design primer, and putative glycerol kinase 3 (BGME16) were selected for functional study. Glycerol kinase 3 might participate in the utilization of glycerol that regulated by JH. RT-PCR assay showed those four genes expressed significantly higher after methoprene application (Fig. 1), and fluctuated during the reproductive cycle in female German cockroaches (Fig. 2), which means that those four genes highly express in normal condition rather than induced by chemical apply. The pattern of fluctuation of four genes are similar with concentration of JH, which keep at low level after emerged, then increase after day 5 and show a peak at day 7, just before virgin female cockroaches form an ootheca at day 8, then immediately drop (Chiang et al., 1994; Treiblmayr et al., 2006).

In order to know the possible function of those four novel genes, we used RNAi to knock-down the gene expression level. First of all, RT-PCR was used to confirm the efficiency of knock-down by RNAi. The results show that the expression level of those four genes significant lower than that of dsEGFP or sham control one day after injection of dsRNA (Fig. 4). In addition, the efficiency remain for five days at least (Fig. 4B).
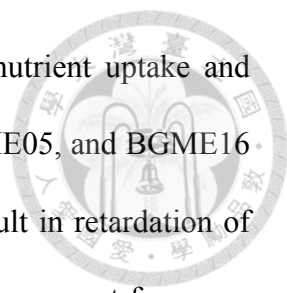
In German cockroach, JH stimulate the ovary development (Belles at al., 1987; Schal et al., 1993; Martin at al., 1995), and the titer of JH coincide with the size of basel oocytes in reproductive cycle (Chiang and Schal, 1994). Base on the success of inhibit

ovary development by RNAi (Fig. 5), BGME02, BGME05, and BGME16 mediate by ovary development JH down-stream regulation.

Previous studies show that the daily activity coincide with JH level in reproduction cycle (Lee and Wu, 1994; Lin and Lee, 1998), and daily locomotor pattern of female German cockroaches is not rhythmic as male cockroaches (Lee and Wu, 1994; Lin and Lee, 1996). In addition, the ovariectomized female German cockroaches could free-run under constant dark condition, and corpora allata (CA) removing reduces the locomotor activity of the ovariectomized female, but the locomotor activity can be recalled by absorb JH analog fenoxycard, which means that ovaries do not affect the locomotor activity, but JH do (Lin and Lee, 1998). Although knock-down the expression level of BGME02, BGME05 and BGME16 by RNAi could inhibit ovary development, RNAi do not affect the rhythmicity of locomotion or activity (Table 2).

Since inhibition of ovary development might be caused by lacking of ovarial component or energy sulpply, the RNAi effect of BGME02, 05, and 16 only on ovary development, but not on locomotion, implies the genes are involve in synthesizing component for ovary development rather than energy supply. However, it is possible that these genes are involving in energy supply, but do not play a critical role in regulation of daily locomotion.

In combination of expression level among various internal organs and silencing effect of RNAi, tentative function of the four target genes can be illustrated (Fig. 3 & 5). It has been found that the daily food intake of female adults increases with JH titer which coincides with the development of ovary (Schal et al., 1994), and the gene expression of Bgl g 1 gene in midgut suggests its involvement in energy and nutrient uptake (Suazo, et al. 2009). Based on the high expression level of BGME07 in fat body,
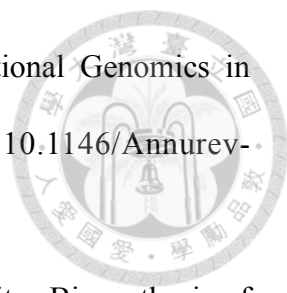
head and leg, but not in ovary implies it might be involving in nutrient uptake and energy supply, but not in ovary development. Since BGME02, BGME05, and BGME16 express higher in fat body than in other organs and their RNAi result in retardation of ovary development, the three genes are related with synthesizing component for ovary development. Although fat body is multi-functional including lipid and carbohydrate storage and utilization, detoxification, synthesis of protein and trehalose (McDougall and Steele, 1988), and amino acid metabolism (Arrese and Soulages, 2010), some of those functions have been confirmed to be regulated by JH in various insect species, such as glycolysis in silkworm (*Bombyx mori*) (Tian et al., 2010), fat-body metabolism in bumblebee (*Bombus terrestris*) (Röseler and Röseler, 1988) and in cockroach *Periplaneta americana*, *Blaberus discoidalis* (Wiens and Gilbert, 1965), synthesis of Vitellogenin (Vg) in burying beetles (*Nicrophorus orbicollis*) (Panaitof and Scott, 2006), in locust (*Locusta migratoria*) (Abu-Hakima, 1981), in mosquito *Aedes aegypti* (Deitsch et al., 1995), in German cockroach (*Blattella germanica*) (Wojchowski et al., 1986, Martın et al., 1996; Comas et al., 2001), and Phospholipid synthesis in cockroach *Leucophaea maderae* (della-Cioppa and Engelmann, 1984). Therefore, these four genes play different roles in ovary development under regulation of JH.

# References

Abuhakima, R. (1981). Vitellogenin Synthesis Induced in Locust Fat-Body by Juvenile-Hormone Analog Invitro. Experientia, 37(12), 1309-1311.

Adams, M. D., Dubnick, M., Kerlavage, A. R., Moreno, R., Kelley, J. M., Utterback, T. R., Nagle, J. W., Fields, C., & Venter, J. C. (1992). Sequence Identification of 2,375 Human Brain Genes. Nature, 355(6361), 632-634. doi: Doi 10.1038/355632a0

Altincicek, B., Knorr, E., & Vilcinskas, A. (2008). Beetle immunity: Identification of immune-inducible genes from the model insect Tribolium castaneum. Dev Comp Immunol, 32(5), 585-595. doi: Doi 10.1016/J.Dci.2007.09.005

Antonio, D. S. M., Guidugli-Lazzarini, K. R., do Nascimento, A. M., Simoes, Z. L. P., & Hartfelder, K. (2008). RNAi-mediated silencing of vitellogenin gene function turns honeybee (Apis mellifera) workers into extremely precocious foragers. Naturwissenschaften, 95(10), 953-961. doi: Doi 10.1007/S00114-008-0413-9

Arakane, Y., Hogenkamp, D. G., Zhu, Y. C., Kramer, K. J., Specht, C. A., Beeman, R. W., Kanost, M. R., & Muthukrishnan, S. (2004). Characterization of two chitin synthase genes of the red flour beetle, Tribolium castaneum, and alternate exon usage in one of the genes during development. Insect Biochemistry and Molecular Biology, 34(3), 291-304. doi: Doi 10.1016/J.Ibmb.2003.11.004

Arrese, E. L., & Soulages, J. L. (2010). Insect fat body: energy, metabolism, and regulation. Annu Rev Entomol, 55, 207-225. doi: 10.1146/annurev-ento-112408-085356

Belles, X. (2010). Beyond Drosophila: RNAi In Vivo and Functional Genomics in Insects. Annu Rev Entomol, 55, 111-128. doi: Doi 10.1146/Annurev-Ento-112408-085301

Belles, X., Casas, J., Messeguer, A., & Piulachs, M. D. (1987). Invitro Biosynthesis of Jh-Iii by the Corpora Allata of Adult Females of Blattella-Germanica (L). Insect Biochemistry, 17(7), 1007-1010.

Burand, J. P., & Hunter, W. B. (2013). RNAi: Future in insect management. Journal of Invertebrate Pathology, 112, S68-S74. doi: Doi 10.1016/J.Jip.2012.07.012

Chang, H. W., & Lee, H. J. (2001). Inconsistency in the expression of locomotor and ERG circadian rhythms in the German cockroach, Blattella germanica (L.). Arch Insect Biochem Physiol, 48(3), 155-166. doi: 10.1002/arch.1068

Chiang, A. S., & Schal, C. (1994). Cyclic Volumetric Changes in Corpus Allatum Cells in Relation to Juvenile-Hormone Biosynthesis during Ovarian Cycles in Cockroaches. Arch Insect Biochem Physiol, 27(1), 53-64. doi: Doi 10.1002/Arch. 940270107

Ciudad, L., Belles, X., & Piulachs, M. D. (2007). Structural and RNAi characterization of the German cockroach lipophorin receptor, and the evolutionary relationships of lipoprotein receptors. BMC Mol Biol, 8, 53. doi: 10.1186/1471-2199-8-53

Ciudad, L., Piulachs, M. D., & Belles, X. (2006). Systemic RNAi of the cockroach vitellogenin receptor results in a phenotype similar to that of the Drosophila yolkless mutant. (Vol 273, pg 325, 2006). Febs Journal, 273(3), 671-671.

Comas, D., Piulachs, M. D., & Belles, X. (2000). Vitellogenin of Blattella germanica (L.) (Dictyoptera, Blattellidae): Nucleotide sequence of the cDNA and analysis of

the protein primary structure. Arch Insect Biochem Physiol, 45(1), 1-11. doi: Doi 10.1002/1520-6327(200009)45:1<1::Aid-Arch1>3.3.Co;2-F

Comas, D., Piulachs, M. D., & Belles, X. (2001). Induction of vitellogenin gene transcription in vitro by juvenile hormone in Blattella germanica. Mol Cell Endocrinol, 183(1-2), 93-100.

Cooper, R. A., & Schal, C. (1992). Effects of Protein Type and Concentration on Development and Reproduction of the German-Cockroach, Blattella-Germanica. Entomologia Experimentalis Et Applicata, 63(2), 123-134.

Cooper, R. A., & Schal, C. (1992). Differential Development and Reproduction of the German-Cockroach (Dictyoptera, Blattellidae) on 3 Laboratory Diets. Journal of Economic Entomology, 85(3), 838-844.
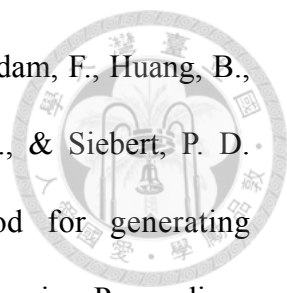
Cusson, M., Yagi, K. J., Guan, X. C., & Tobe, S. S. (1992). Assessment of the Role of Cyclic-Nucleotides in Allatostatin-Induced Inhibition of Juvenile-Hormone Biosynthesis in Diploptera-Punctata. Mol Cell Endocrinol, 89(1-2), 121-125.
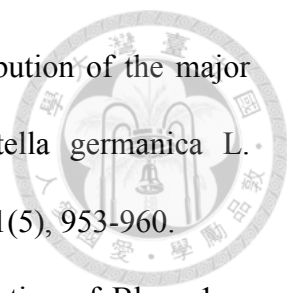
Degliesposti, M., Devries, S., Crimi, M., Ghelli, A., Patarnello, T., & Meyer, A. (1993). Mitochondrial Cytochrome-B - Evolution and Structure of the Protein. Biochimica Et Biophysica Acta, 1143(3), 243-271.
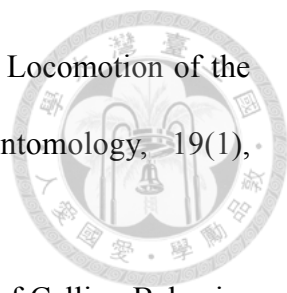
Deitsch, K. W., Chen, J. S., & Raikhel, A. S. (1995). Indirect Control of Yolk Protein Genes by 20-Hydroxyecdysone in the Fat-Body of the Mosquito, Aedes-Aegypti. Insect Biochemistry and Molecular Biology, 25(4), 449-454.
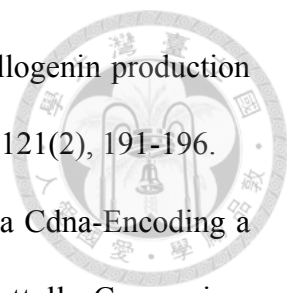
Dellacioppa, G., & Engelmann, F. (1984). Phospholipid-Synthesis in the Fat-Body Endoplasmic-Reticulum during Primary and Secondary Juvenile-Hormone Stimulation of Vitellogenesis in Leucophaea-Maderae. Wilhelm Rouxs Archives of Developmental Biology, 193(2), 78-85.
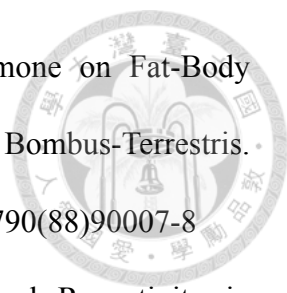
Diatchenko, L., Lau, Y. F. C., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E. D., & Siebert, P. D. (1996). Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proceedings of the National Academy of Sciences of the United States of America, 93(12), 6025-6030. doi: Doi 10.1073/Pnas.93.12.6025

Dietzl, G., Chen, D., Schnorrer, F., Su, K. C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S., Couto, A., Marra, V., Keleman, K., & Dickson, B. J. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature, 448(7150), 151-156. doi: 10.1038/nature05954

Dong, Y., & Friedrich, M. (2005). Nymphal RNAi: systemic RNAi mediated gene knockdown in juvenile grasshopper. Bmc Biotechnology, 5. doi: Artn 25

Doi 10.1186/1472-6750-5-25

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature, 391(6669), 806-811. doi: 10.1038/35888

Gadot, M., Burns, E., & Schal, C. (1989). Juvenile-Hormone Biosynthesis and Oocyte Development in Adult Female Blattella-Germanica - Effects of Grouping and Mating. Arch Insect Biochem Physiol, 11(3), 189-200. doi: Doi 10.1002/Arch.940110306

Galzie, Z., Kinsella, A. R., & Smith, J. A. (1997). Fibroblast growth factors and their receptors. Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire, 75(6), 669-685. doi: Doi 10.1139/Bcb-75-6-669

Gore, J. C., & Schal, C. (2004). Gene expression and tissue distribution of the major human allergen Bla g 1 in the German cockroach, Blattella germanica L. (Dictyoptera : Blattellidae). Journal of Medical Entomology, 41(5), 953-960.

Gore, J. C., & Schal, C. (2005). Expression, production and excretion of Bla g 1, a major human allergen, in relation to food intake in the German cockroach, Blattella germanica. Medical and Veterinary Entomology, 19(2), 127-134. doi: Doi 10.1111/J.0269-283x.2005.00550.X

Huvenne, H., & Smagghe, G. (2010). Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. Journal of Insect Physiology, 56(3), 227-235. doi: Doi 10.1016/J.Jinsphys.2009.10.004

Irles, P., Belles, X., & Piulachs, M. D. (2009). Identifying genes related to choriogenesis in insect panoistic ovaries by Suppression Subtractive Hybridization. BMC Genomics, 10, 206. doi: 10.1186/1471-2164-10-206

Kennerdell, J. R., & Carthew, R. W. (1998). Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. Cell, 95(7), 1017-1026. doi: Doi 10.1016/S0092-8674(00)81725-0

Khalil, S. M. S., Anspaugh, D. D., & Roe, R. M. (2006). Role of juvenile hormone esterase and epoxide hydrolase in reproduction of the cotton bollworm, Helicoverpa zea. Journal of Insect Physiology, 52(7), 669-678.

Lee, C. M., Su, M. T., & Lee, H. J. (2009). Pigment Dispersing Factor: An Output Regulator of the Circadian Clock in the German Cockroach. Journal of Biological Rhythms, 24(1), 35-43.

Lee, H. J., & Wu, Y. L. (1994). Mating Effects on the Feeding and Locomotion of the German-Cockroach, Blattella-Germanica. Physiological Entomology, 19(1), 39-45. doi: Doi 10.1111/J.1365-3032.1994.Tb01071.X

Liang, D. S., & Schal, C. (1994). Neural and Hormonal-Regulation of Calling Behavior in Blattella-Germanica Females. Journal of Insect Physiology, 40(3), 251-258.

Lin, T. M., & Lee, H. J. (1996). The expression of locomotor circadian rhythm in female German cockroach, Blattella germanica (L). Chronobiology International, 13(2), 81-91. doi: Doi 10.3109/07420529609037072

Lin, T. M., & Lee, H. J. (1998). Parallel control mechanisms underlying locomotor activity and sexual receptivity of the female German cockroach, Blattella germanica (L.). Journal of Insect Physiology, 44(11), 1039-1051. doi: Doi 10.1016/S0022-1910(98)00069-9

Lum, L., Yao, S., Mozer, B., Rovescalli, A., Von Kessler, D., Nirenberg, M., & Beachy, P. A. (2003). Identification of Hedgehog pathway components by RNAi in Drosophila cultured cells. Science, 299(5615), 2039-2045. doi: 10.1126/science.1081403

Maestro, J. L., & Belles, X. (2006). Silencing allatostatin expression using double-stranded RNA targeted to preproallatostatin mRNA in the German cockroach. Arch Insect Biochem Physiol, 62(2), 73-79. doi: Doi 10.1002/Arch.20123

MartÍN, David, Piulachs, Maria-Dolors, & BellÉS, Xavier. (1995). Patterns of haemolymph vitellogenin and ovarian vitellin in the German cockroach, and the role of Juvenile Hormone. Physiological Entomology, 20(1), 59-65. doi: 10.1111/j.1365-3032.1995.tb00801.x

Martin, D., Piulachs, M. D., & Belles, X. (1996). Inhibition of vitellogenin production by allatostatin in the German cockroach. Mol Cell Endocrinol, 121(2), 191-196.

Martinezgonzalez, J., & Hegardt, F. G. (1995). Characterization of a Cdna-Encoding a Cytosolic Peptidylprolyl Cis-Trans-Isomerase from Blattella-Germanica. European Journal of Biochemistry, 234(1), 284-292. doi: Doi 10.1111/J. 1432-1033.1995.284_C.X

Mcdougall, G. E., & Steele, J. E. (1988). Free Fatty-Acids as a Source of Energy for Trehalose Synthesis in the Fat-Body of the American Cockroach (Periplaneta-Americana). Insect Biochemistry, 18(6), 591-597. doi: Doi 10.1016/0020-1790(88)90011-X

Panaitof, S. C., & Scott, M. P. (2006). Effect of juvenile hormone on vitellogenin gene expression in the fat body of burying beetles, Nicrophorus orbicollis. Arch Insect Biochem Physiol, 63(2), 82-91. doi: 10.1002/arch.20144

Pridgeon, J. W., & Liu, N. (2003). Overexpression of the cytochrome c oxidase subunit I gene associated with a pyrethroid resistant strain of German cockroaches, Blattella germanica (L.). Insect Biochemistry and Molecular Biology, 33(10), 1043-1048. doi: Doi 10.1016/S0965-1748(03)00120-6

Pridgeon, J. W., Zhang, L., & Liu, N. N. (2003). Overexpression of CYP4G19 associated with a pyrethroid-resistant strain of the German cockroach, Blattella germanica (L.). Gene, 314, 157-163. doi: Doi 10.1016/S0378-1119(03)00725-X

Robich, R. M., Rinehart, J. P., Kitchen, L. J., & Denlinger, D. L. (2007). Diapause-specific gene expression in the northern house mosquito, Culex pipiens L., identified by suppressive subtractive hybridization. Journal of Insect Physiology, 53(3), 235-245. doi: Doi 10.1016/J.Jinsphys.2006.08.008

Roseler, P. F., & Roseler, I. (1988). Influence of Juvenile-Hormone on Fat-Body Metabolism in Ovariolectomized Queens of the Bumblebee, Bombus-Terrestris. Insect Biochemistry, 18(6), 557-563. doi: Doi 10.1016/0020-1790(88)90007-8

Schal, C., & Chiang, A. S. (1995). Hormonal-Control of Sexual Receptivity in Cockroaches. Experientia, 51(9-10), 994-998.

Schal, C., Holbrook, G. L., Bachmann, J. A. S., & Sevala, V. L. (1997). Reproductive biology of the German cockroach, Blattella germanica: Juvenile hormone as a pleiotropic master regulator. Arch Insect Biochem Physiol, 35(4), 405-426. doi: Doi 10.1002/(Sici)1520-6327(1997)35:4<405::Aid-Arch5>3.0.Co;2-Q

Scharrer, B. (1987). Insects as Models in Neuroendocrine Research. Annu Rev Entomol, 32, 1-16.

Schluns, H., & Crozier, R. H. (2007). Relish regulates expression of antimicrobial peptide genes in the honeybee, Apis mellifera, shown by RNA interference. Insect Molecular Biology, 16(6), 753-759.

Seitz, V., Clermont, A., Wedde, M., Hummel, M., Vilcinskas, A., Schlatterer, K., & Podsiadlowski, L. (2003). Identification of immunorelevant genes from greater wax moth (Galleria mellonella) by a subtractive hybridization approach. Dev Comp Immunol, 27(3), 207-215.

Sokolove, Phillip G., & Bushell, Wayne N. (1978). The chi square periodogram: Its utility for analysis of circadian rhythms. Journal of Theoretical Biology, 72(1), 131-160. doi: http://dx.doi.org/10.1016/0022-5193(78)90022-X

Suazo, A., Gore, C., & Schal, C. (2009). RNA interference-mediated knock-down of Bla g 1 in the German cockroach, Blattella germanica L., implicates this allergen-

encoding gene in digestion and nutrient absorption. Insect Molecular Biology, 18(6), 727-736. doi: Doi 10.1111/J.1365-2583.2009.00912.X
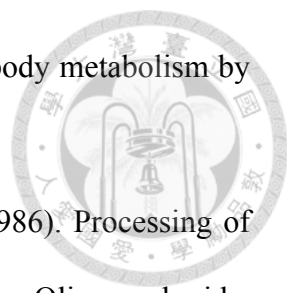
Suren-Castillo, S., Abrisqueta, M., & Maestro, J. L. (2012). FoxO inhibits juvenile hormone biosynthesis and vitellogenin production in the German cockroach. Insect Biochemistry and Molecular Biology, 42(7), 491-498. doi: Doi 10.1016/ J.Ibmb.2012.03.006

Suzuki, Y., Truman, J. W., & Riddiford, L. M. (2008). The role of broad in the development of Tribolium castaneum: implications for the evolution of the holometabolous insect pupa. Development, 135(3), 569-577. doi: Doi 10.1242/ Dev.015263

Terenius, O., Papanicolaou, A., Garbutt, J. S., Eleftherianos, I., Huvenne, H., Kanginakudru, S., Albrechtsen, M., An, C., Aymeric, J. L., Barthel, A., Bebas, P., Bitra, K., Bravo, A., Chevalier, F., Collinge, D. P., Crava, C. M., de Maagd, R. A., Duvic, B., Erlandson, M., Faye, I., Felfoldi, G., Fujiwara, H., Futahashi, R., Gandhe, A. S., Gatehouse, H. S., Gatehouse, L. N., Giebultowicz, J. M., Gomez, I., Grimmelikhuijzen, C. J., Groot, A. T., Hauser, F., Heckel, D. G., Hegedus, D. D., Hrycaj, S., Huang, L., Hull, J. J., Iatrou, K., Iga, M., Kanost, M. R., Kotwica, J., Li, C., Li, J., Liu, J., Lundmark, M., Matsumoto, S., Meyering-Vos, M., Millichap, P. J., Monteiro, A., Mrinal, N., Niimi, T., Nowara, D., Ohnishi, A., Oostra, V., Ozaki, K., Papakonstantinou, M., Popadic, A., Rajam, M. V., Saenko, S., Simpson, R. M., Soberon, M., Strand, M. R., Tomita, S., Toprak, U., Wang, P., Wee, C. W., Whyard, S., Zhang, W., Nagaraju, J., Ffrench-Constant, R. H., Herrero, S., Gordon, K., Swevers, L., & Smagghe, G. (2011). RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications

for experimental design. Journal of Insect Physiology, 57(2), 231-245. doi: 10.1016/j.jinsphys.2010.11.006

Tian, L., Guo, E., Wang, S., Liu, S., Jiang, R. J., Cao, Y., Ling, E., & Li, S. (2010). Developmental regulation of glycolysis by 20-hydroxyecdysone and juvenile hormone in fat body tissues of the silkworm, Bombyx mori. J Mol Cell Biol, 2(5), 255-263. doi: 10.1093/jmcb/mjq020

Tobe, S. S., & Stay, B. (1985). Structure and Regulation of the Corpus Allatum. Advances in Insect Physiology, 18, 305-432.

Treiblmayr, K., Pascual, N., Piulachs, M. D., Keller, T., & Belles, X. (2006). Juvenile hormone titer versus juvenile hormone synthesis in female nymphs and adults of the German cockroach, Blattella germanica. J Insect Sci, 6, 1-7. doi: 10.1673/031.006.4301

Tsai, C. W., & Lee, H. J. (2000). Circadian locomotor rhythm masked by the female reproduction cycle in cockroaches. Physiological Entomology, 25(1), 63-73. doi: Doi 10.1046/J.1365-3032.2000.00165.X

Ursic-Bedoya, R. J., & Lowenberger, C. A. (2007). Rhodnius prolixus: Identification of immune-related genes up-regulated in response to pathogens and parasites using suppressive subtractive hybridization. Dev Comp Immunol, 31(2), 109-120. doi: Doi 10.1016/J.Dci.2006.05.008

Vogel, H., Badapanda, C., & Vilcinskas, A. (2011). Identification of immunity-related genes in the burying beetle Nicrophorus vespilloides by suppression subtractive hybridization. Insect Molecular Biology, 20(6), 787-800. doi: Doi 10.1111/J.1365-2583.2011.01109.X

Wiens, A. W., & Gilbert, L. I. (1965). Regulation of cockroach fat-body metabolism by the corpus cardiacum in vitro. Science, 150(3696), 614-616.

Wojchowski, D. M., Parsons, P., Nordin, J. H., & Kunkel, J. G. (1986). Processing of Pro-Vitellogenin in Insect Fat-Body - a Role for High-Mannose Oligosaccharide. Developmental Biology, 116(2), 422-430. doi: Doi 10.1016/0012-1606(86)90143-0

Xu, W. N., & Han, Z. J. (2008). Cloning and phylogenetic analysis of sid-1-like genes from aphids. J Insect Sci, 8.

Yu, N., Christiaens, O., Liu, J. S., Niu, J. Z., Cappelle, K., Caccia, S., Huvenne, H., & Smagghe, G. (2013). Delivery of dsRNA for RNAi in insects: an overview and future directions. Insect Science, 20(1), 4-14. doi: Doi 10.1111/J. 1744-7917.2012.01534.X

Yu, Q., Lin, T. H., Feng, G. Z., Yang, K., & Pang, Y. (2008). Functional analysis of the putative antiapoptotic genes, p49 and iap4, of Spodoptera litura nucleopolyhedrovirus with RNAi. Journal of General Virology, 89, 1873-1880. doi: Doi 10.1099/Vir.0.2008/001008-0

Zhang, C., & Xia, Y. (2009). Identification of genes differentially expressed in vivo by Metarhizium anisopliae in the hemolymph of Locusta migratoria using suppression-subtractive hybridization. Curr Genet, 55(4), 399-407. doi: 10.1007/s00294-009-0254-x

Zhou, X. G., Wheeler, M. M., Oi, F. M., & Scharf, M. E. (2008). RNA interference in the termite Reticulitermes flavipes through ingestion of double-stranded RNA. Insect Biochemistry and Molecular Biology, 38(8), 805-815.

Zhu, J. S., Busche, J. M., & Zhang, X. (2010). Identification of juvenile hormone target genes in the adult female mosquitoes. Insect Biochemistry and Molecular Biology, 40(1), 23-29. doi: Doi 10.1016/J.Ibmb.2009.12.004

Zhu, Y., Johnson, T. J., Myers, A. A., & Kanost, M. R. (2003). Identification by subtractive suppression hybridization of bacteria-induced genes expressed in Manduca sexta fat body. Insect Biochem Mol Biol, 33(5), 541-559.
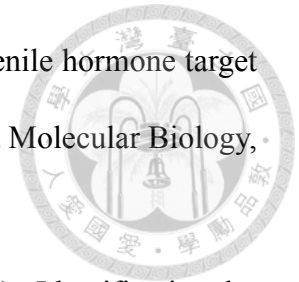
# Appendix

**Primer list**

The primers used in experiments were designed by Primer3 software (http://frodo.wi.mit.edu/), in order to select an optimal primer annealing temperature of 60°C.

| Primer name | | Primer sequence | Length of product (bp) |
|---|---|---|---|
| BGME02 | F | 5' CATCTTCAGATGTTAGAAATAGCGTA 3' | 171 |
| | R | 5' ACCGGTGAGGTAAAAGCTGA 3' | |
| BGME02 (for qRT-PCR) | F | 5' CTGCGCGAGAATTGCTGTGTTTCT 3' | |
| | R | 5' AAAGCTGACACATGACACTCGC 3' | |
| BGME05 | F | 5' TGTCCAATGACTGAATTCTCTTG 3' | 202 |
| | R | 5' CGGAAGTGTTGGATACCAGAA 3' | |
| BGME05 (for qRT-PCR) | F | 5' CTGGTATCCAACACTTCCGTCA 3' | 199 |
| | R | 5' ATGGCTAACTGCTTCTGCTCCAAGA 3' | |
| BGME07 | F | 5' TTTGTTTTTCCATCGCATCA 3' | 181 |
| | R | 5' CGGTTTCGTTTACGAATGTGT 3' | |
| BGME016 | F | 5' CGTGGACCTTCGTATCTGCT 3' | 193 |
| | R | 5' CAGCTGCGTCTTGTCTCTTG 3' | |
| BG_Actin_2 | F | 5' TCTGGATCTGGCTGGTCGTGATTT 3' | |
| | R | 5' TCGAAGTCGAGGGCAACATAGCAA 3' | |
| BGME02_T7 (for dsRNA synthesis) | F | 5' TAA TAC GAC TCA CTA TAG GGA GAC ATC TTC AGA TGT TAG AAA TAG CGT A 3' | |
| | R | 5' ACC GGT GAG GTA AAA GCT GAA GAG GGA TAT CAC TCA GCA TAA T 3' | |
| BGME05_T7 (for dsRNA synthesis) | F | 5' TAA TAC GAC TCA CTA TAG GGA GAT GTC CAA TGA CTG AAT TCT CTT G 3' | |
| | R | 5' CGG AAG TGT TGG ATA CCA GAA AGA GGG ATA TCA CTC AGC ATA AT 3' | |

BGME07_T7
(for dsRNA
synthesis)

F  5' TAA TAC GAC TCA CTA TAG GGA GAT
TTG TTT TTC CAT CGC ATC A 3'

R  5' CGG TTT CGT TTA CGA ATG TGT AGA
GGG ATA TCA CTC AGC ATA AT 3'

BGME16_T7
(for dsRNA
synthesis)

F  5' TAA TAC GAC TCA CTA TAG GGA GAC
GTG GAC CTT CGT ATC TGC T 3'

R  5' CAG CTG CGT CTT GTC TCT TGA GAG
GGA TAT CAC TCA GCA TAA T 3'

**Sequences of gene fragments obtained via SSH in female German cockroaches after topical application of methoprene (juvenile hormone analog).**

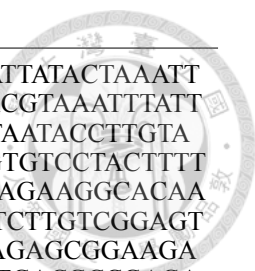| | |
|---|---|
| BGME01_1 | GATTAGCGTGGTCGCGGCCGAGGTGCCAGGTTGGTTTCTATCTATAATAAAAT<br>TTAATATTTTA |
| BGME01_2 | GATTAGCGTGGTCGCGGCCGAGGTATAAGACTAAATTACCTTAGGGATAACAG<br>CGTAATCTTTTTTGAGAGTTCTTATCAATAAAAGGGGGTTGCGACCTCGATGTT<br>GGATTAAGATTAACATTAGGTGTAGAAGTTTAATTTGTTAGGTCTGTTCGACCT<br>TTAAAATCTTACATGATCTGAGTTCAAACCGGCGTGAGCCAGGTTGGTTTCTA<br>TCTATAATAAAATTTAATATTTTA |
| BGME01_3 | GATTTCGAGCCGGCCGCCCGGGCAGGTCTTTTTTGAGAGTTCTTATCAATAAA<br>AGGGGTTGCGACCTCGATGTTGGATTAAGATTAACATTAGGTGTAGAAGTTTA<br>ATTTGTTAGGTCTGTTCGACCTTTAAAATCTTACATGATCTGAGTTCAAACCGG<br>CGTGAGCCAGGTTGGTTTCTATCTATAATAAAATTTAATATTTTA |
| BGME01_4 | AAGCTTTTTTTTTTTATTTTGATTCTTTGGACATCCAGAAGTTTATATTTTAATTT<br>TACCAGGGTTTGGTATGATTTCTCATATCATTTGCCATGAAAGAGGTAAAAAG<br>GAAGCTTTTGGAAATTTAGGAATAATTTTTGCTATATTAGCAATTGGTTTATTAG<br>GATTTGTTGTTCGAGCTCATCATATATTTACTGTAGGAATAGACGTGGATACCC<br>GAGCCTATTTTACTTCAGCTACTATAATTATTGCTGTACCTCGGCCGCGACCAT<br>CGAGCGGCCGCCCGGGCAGGTACTAAAATATTAAATTTTATTATAGATAGAAA<br>CCAACCTGGCTCACGCCGGTTTGAACTCAGATCATGTAAGATTTTAAAGGTCG<br>AACAGACCTAACAAATTAAACTTCTACACCTAATGTTAATCTTAATCCAACATC<br>GAGGTCGCAACCCCTTTTATTGATAAGAACTCTCAAAAAAGATTACGCTGTTA<br>TCCCTAAGGTAATTTAGTCTTATAATCATTATAAATGGATCAAAATTCTATAAAT<br>TAATATTTATTAATAAAAAGAGTTATTTATATCTTCCTGTCACCCCAACAAAAA<br>ATTATAATTAATATTGCGAAACAAAACTAATCTAAAAATTATAATATTAAACTCT<br>ATAGGGTCTTCTCGTCCCATAACAATATTTAAGCTTTTTTACTTAATAATTAAAT<br>TCTAATAAAATAATGAGATAGCTCATATTTCGTCCAACCATTCATTCCAGCCT<br>CTAATTAAGAAACTAATGATTATGCTACCTTTGCACGGTCAGGTTACCGCGGC<br>CCTTCAAAACCTCGGCCGCGACCACGCTAATCGAATTCCCGCGGCCGCCATG<br>CGGCCGGAGCTGGATGAGCTGATCAGCTAAAGTT |
| BGME02 | TTATATTTCTTTGTAAATGAAAATAGACACATCTTCAGATGTTAGAAATAGCGT<br>ATACTGAAGACATAATTTTGTTATAACTGGGGAGGTAAGACATGACACATGAG<br>CTGCGCGAGAATTGCTGTGTTTCTCCGTTACTATGAACAGAAGAATGCTACTG<br>CGCATCGCGAGTGTCATGTGTCAGCTTTTACCTCACCGGTTCTACATCCAAAA<br>TATTTTTGTTATTTATGTTTATTATATTTCTGTAATAGTAGAATTCAATCAATGTAT<br>TTTGTTTATAGAGAATTATAAATTACTTTCAAATCAAAAAAAAAAAAAAAAAAAA<br>AAAAAAAAAAAAAAAAGCTT |
| BGME03 | ACAACATTGCCATGCTACTAATAAACTTAGGAAGAACATTAAATTTCACTGTAT<br>TCCAAAGGGACCAATTGCTGAACACTGGCAGAACGTGGTGAGGAAAGGCAT<br>AAATCCTGATTTCACAAAGAAGACTGTCGACAAAGAATTGCCTGTAAACATC<br>CCTCTCAAGTGCGTAATCAATTAAGTAGTGTTCACAAAAAGACGTGGTTTAAT<br>GACAATCGAATAAACATTTTGATATATGTTACAATAAGTTAAACATATAAAATT<br>GTATAACATTATTTATAAATAATAAATTTATTGCATTTAATACTAAAAAAAAAAA<br>AAAAAAAAAAAAAAAAAAAAGCTT |
| BGME04 | AGAGACCTGAGTGTGTCAGGAGCTGTGACGCAATGCTATCGTGACATGGGAG<br>CTCGTCACAGGGCCCGAGCACATTCTATCCAGATCATTAAGGTGGAGAAGGT<br>GAAGGCCGCAAACTGCAGGCGTCCTTTGGTGAAACAATTTCACAACTCCAAG<br>ATCAGATTCCCCTTACCAAAGCGCATTCAACAGCGCAACCTGTTGCCACGCTT<br>CTCTGTAAGAAAGCCACGAACTTACTTTATGTAAATTTGTTAAAAGAAAATAA<br>ATATTTTGCAGAAATGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA<br>AAAAAAAGCTT |

36

| | |
|---|---|
| BGME05 | TAAATAATGTGTCCAATGACTGAATTCTCTTGTAGAATATACAAAACATTTTAC<br>AGCAATCAATCATTTTCATTACACAAACTTGGGTAATATAGAGAATATGTGCCA<br>AATTCTGTCTTAAATAAAACAGTATTCAGATCTACTATGTGATGTGAGTATACA<br>GTTATTTTAATACCTTGGAACGTATTCATTCTGGTATCCAACACTTCCGTCATAA<br>CTACAAAAAACATTTAATGATTTTTTATGCATTCTCGCTTTGGTATAGATTTAGC<br>TGTCTTGGAGCAGAAGCAGTAGCCATTGGTTTATTTTAACTTTTTAAAATTATC<br>ACTATTTCAAATAAATAGCTWCTCTCATTTCTAGTCTTAACTATATAACGAAGA<br>AAAACAATTATTAATAACTTAATTTGATCTGAGCAAATACATGGATATTACATTA<br>ATCGAACTCTGAAATTTCACAGTTCAGAAATTTGTACTTTCTAACATTAAAATA<br>TCTGAGAGCATCTCATGGAACATGTGAAGAAATTAATGATTGCTTGAAAGCAC<br>AATCACTATCACGCATTGTTCTAATGAAAATATATTAACAAAAATTATTAAATTA<br>CATATTAATGTTTTTATGAATCACAATAATTCTCTTAGAGAAAATTTCTGATTGT<br>AGAATTTCGGTAACATGAATAGATGGATGAAATGAAAACTAGGAAAGCGGCT<br>ATAGTACCTTGGTATATGAAGGCAGTGAATTGCTAAAATAAAAGCTTTCATCCA<br>ATGTC |
| BGME07 | GATTAGCGTGGTCGCGGCCGAGGTTTACGGGTTAAGCATGTGAGCGGTCACT<br>TCCTTGAAACGTAATATTACGGTCTTAGCAGCGAAAGGGTTAAGAATTTGTTT<br>TTCCATCGCATCATATTAAGTCATTTCATGTTACTTCATTGTAATCCCAGCTTAA<br>GACTTTATTTATTTTTCTTTTTCAGTTGGTAATAAAGTCAGGTGAAAAGGGTTT<br>ATATTTGGGTGCCAGTGTCGTCAGAATATTTTGTAATTGCTGTTACACATTCGT<br>AAACGAAACCGTTGAAAAATAAAATTTGCTTAAAAAAAAAAAAAAAAAAAAAA<br>AAAAAAAAAAAAGCTT |
| BGME08 | ACCCTCTGAGCTGCTCCGGCCGCCATGGCGGCCGCGGGATTCGATTTCGAGC<br>GGCCGCCCGGGCAGGTGTCGCAACAGGAATGGTAAGAGGGTAGTTCTTCCTG<br>TTGAAATCCCAATACAATGCGAACCTGTTTTAAACTGAAATCCGATTCTTGTTA<br>ATGGACTTAATTTAAAAAAATCTGACTTATGAAGAAAACTGGAATATTTTCAAA<br>AATTGAACTATTTATGTATTTAATAAATAAAGAATGATAGCAACTCGAAAAAAA<br>AAAAAAAAAAAAAAAAAAAAAAAAGCTT |
| BGME09 | GATTCACACTCCTAGCGCTCAGTCTCTCTCTCTCCTCTTCTCTCATGATAAATC<br>AAGCAAGTGGCCCTAGGCAGCCCTCCAGGAGGAGAAGCCTTTAAGTGCAGA<br>ACAAAATCAGCATATTCCCATCCAAGCCTCCATCATACATTATGCATGCGACAA<br>AGTTTGGCAACAGTGGAGCTGATGTGATGACACAGCGAATCAATAAGAACCC<br>ACCGCGTGAAACCTTTATAGTGGTTACTTTTTTAAGGGCTCAAGTGAAGAAAT<br>TGTCTTTGTCAACTTTGGCTTAGAATATCCTTATACAGTTCAGGTACTTTGTCG<br>GGGTCCACTGGTCTAGGTAAAAAATGTGTGAATTCTGGTGCCGTTTAGTCCT<br>AGTCCCTTCTCTCGGGGTCCCATCTTTATTTAATGCAACATAGTATCGCCTTCC<br>AGTGTCCACGTGCTTATATAGGTTTGACGAGTACGTATTATACCAGTTTTCTTC<br>GAACTGTTCTCTGAATACACACTCTTGGGTTAGTTTTTCCTGTAAAAAGAAAA<br>AGAGGGAGAGCATTAGGAAAAATAGCTAATGCATGCTGGGCTTAAAATC |
| BGME10 | TTAGCGTGGTCGCGGCCGAGGTACACGCCTCCACAATATTTAACTGGTTAGTG<br>TTTGGTAGCACATTGAATGTCAAAGCAGGCCAGAAAACATGGATTGTGGTGG<br>TGGTAGTGGTGAAGTGTTCATTCAATTCAAAGCCAAGGATTTGGGGGACTTCT<br>CATTCTTTTTTCTTCTTCTTCAAACATAATAATCATAATAATAGTAACCATTAAT<br>GAGAGAGTCTCTGTTCAAGATTTACACTCATTTGATGATGNCACCTAAAAAAA<br>GGAAAACACTGAAAAATGTCCAACAAATTTCCAAAACAATNTACCTGCCCGG<br>GCGGCCGCTCGAAATCACGAATTTCTGGATTCCGATACGTAACGCTNTGCAGC<br>TGCTT |

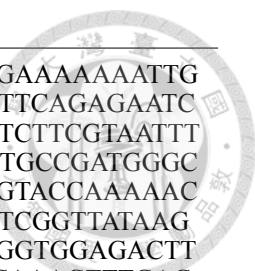| | |
|---|---|
| BGME11 | TCTTCCCAGTCTCCAGTCGCTTACTAACAGGACTGAGCTCACGACGCTTGCG GTGTGAACGTAGGAACGATGGCGGACGAGCCGGCGCCAGAAGCTCCACCTG CAGAGGCAGCACCAGCTGAAAGCCTGCCACCACCACAGGCTTCCAAGAGCC GTGACCCCAACCGCACTATCCTATTCAGACACTGGAGCCGTCCGACTCGCTTG CAGTACAGATATCTGTATGACTACCGCCACAATTACTACGATGATGTAATTGAC TACCTGGACAGGAAGACAAAGGGYATCTCCCGGGACATCCCTAGGGCACAGA CTTGGGCTGAGCGCATGCTTCGCACATATAATAAGGACTTAACATCAGCAGCC AACACAAGCAGATCAAGGGACATTGAACTCATCAGCAACATCCGTGCCAGCA CCAATGTCTATAACTACCACAGCAAGGATTTCCTCTACAGGAAATATCCAGCT CTTGCTCTTTGAATGAAGAAACAAACTGGCAATGTGTGGGGTGTACCTGAAT TAGAATGACGAAAGAAACCAGTAAATTTTTAACTGAACTATCCCTTTATCTG CTTGGCTCATACTAAAACTTGTAAGAATGTTCTTTCACCCCACTCTTGAGGAC TCAAAGCAATTTTCCAATGACAATTTTTCATTGAGTTTGCCTTTGAAGGATGTC ATGTGCAGTCTTCAATGACTATAGAATGAAATGAACATCTCTTCATTTTTATGT AATTTTCTTCTTTAACTGTTTTATGTTCAACACGAATATTTAAATTGGAACTTAT GAACTATCCTCAGATGATTTGAAATTCCTGATGATTATAGTTGTCTTAGATTTGT AGGGTGAAAGGAGTATATTTGATTTTCAAGTCATGATTGTGTACAGTTCTTCAT CCATTGTATCACTGCCGAAGTTGATATTCACCCATTGAACTATACAATCCTAAT AAAACATCATTTAGACACTGTATGAAACTAAGGATGACAAATGTATACTTGTC ATTTRRAATAATAATGTCACAATACTAATRAMAGAAAAAAAAAAAAAAAAAAA AARWAAGTACTACTAG |
| BGME13 | TTCGAGCGGCCGCCCGGGCAGGTACTTCCTAATATAAAATTTACTTCAGTAAA TTACAGTAAATGATAATTTGAAATTTTTTATATTTGATGATTCTTCAGACTAGCA GATCCAAACTGAGGATCCCCAAAAGCCCATTCAGCGGTGGATGAGGTGATGG ATACCAGGGATATCCAGGGTAGTATGGTTTCGATTCTGCCACCAGGATCATTGC GCCTAGCGCAATGAGTAAAACAAACAGGGAAGTCTTAGCATTCATTGTATTAC CTCGGCCGCGACCACGCTAAGGGCGANTTCGCGCCGAA |
| BGME14 | AAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACTTAATTAAGTTATCTCAAATTT TAGTTGTTGCTGGATATAAAATATAATATAGGAAATATAAAACAGTTAAAAGTT GACCTGTAATAATATATGGTTCTTCAACAGGTCGAGCTCCAATTCATGTTAATA AAATTACAATATTAACTATAATTCAAAATAGAACTTGGTTGATAGGATAAAATT GTATTCCCCGGAAATTAGAAGTGTATAAAGGTAAAATAAATAAGATTGCAATT GATATTACTAAAGCTATTACTCCTCCTAATTTATTAGGAATTGATCGTAAAATTG CGTAAGCGAATAGGAAATATCATTCAGGTTGAATATGGACTGGTGTAACAAGA GGATTTGCAGGGGTAAAATTATCTGGATCTCCTAAAATGTAAGGTTCCTTAAG TGTTAGAATTGTTAATAATATAATTAATACAATAAAACCAAATGTATCTTTAATT GAAAAATAAGGATGAAATGGAATTTTATCAATATTTCTATTTAATCCTAAAGGA TTATTTGACCCTGTTTGATGAAGAAATAATAAATGAATTAAAACTATTGCACCT GCCCCGGGCGGCCGCTCGAAATC |
| BGME15 | TATTGGCGAAGTCGCATGCTCCGGCCGCCATGGCGGCCGCGGGAATTCGATTT CGAGCGGCCGCCCGGGCAGGTCTTAAAATTTTATTGATAATATTTGGATCTTAA ATGTTTAATTCTGATATTTCAAATTTATAATCGTGGTAATTGGAACTTTAAAACT TTCACTCCTCATGTGACAAATTATTTACGCTGTTAAATGTTTTCATAAACTACA CGAACATTGTTTCATTTCATGTAACTTAAGTAAAATGTATAACATTATGTATGTC ACTTGTATTCACTTTTACATTTTTTATTGAAGCTGCTGAAATTCATTCTTTATGC GTTTGTATTGTTTGCTTACTTTTTATTAAATCAATAAAGACGTGCTGATGACAA AAAAAAAAAAAAAAAAAAAAACTTGAACCTCGGCCGCGACCACCCTAATCA CTATTGAATTCACGGAATTCTGCAGGTCGACCATATGGGAGAGCTCCCAAAGC GTTGGATGCATAGGATGAGTATTCTATATAGTCTCCTAAATAGCCTGGCGGCTC GGGGTAATAGCTGTTTCCTGTGTGAAATGGGTATCCGCTCACAATTCCACACA ACTACGAACCGACAGCCCAAATGTTAAGACTGTGGTGCCTAGGACCGAATAA TTACATTATTGCTTGCTGCTTGCCCGATTGCCAACTTTAAGCCTGGCAAGCTGC TGGATTAATGAAT |

| | |
|---|---|
| BGME16 | TTTTTTGAACGCACTATATTTAATATGCTGTGTATATGGAATTATTATACTAAATT<br>ATAACTTATTCGGATATAATTAGATACACGTGTTATATTTCTAACGTAAATTTATT<br>GTTTTATATGCTCGTTATTGTGAATACTCGCTCTTTTTATAAGTAATACCTTGTA<br>CGTTCGGCAATGATTAAAATCGATCGTCAGTGGAGGTTATGGTGTGTCCTACTTTT<br>GTGGATTTCTGTTTCTGAAGTGGATTTGAGAGAAGTAATATAAGAAGGCACAA<br>TGCCCGTTTCGTCAACCACAGCAAACAAGAAGAAGGATGCTCTTGTCGGAGT<br>CATCGTGGAAGGCACAGAGGAGGCGCGATTTGCGGTGTACAGAGCGGAAGA<br>GAGCGAGGAGTTGGTGTCGCATCGCATCTACATAAAGCAGATGACGCCGAGA<br>GAAGGCTGGTTGGAGCACGACCCGATGGAGATATTGCAGGCCGTGAACGAGT<br>GCATCAGGGCCGGGGTGGAGGCCCTGGGAGAAGCTCAACTACGGCGTGGAG<br>GACATCGTGACGATCGGGATCTCGAACCAGCGGGAGACGACGATCGCCTGGG<br>ACGTGTCGACAGGGGAGCCGCTCTACAACGCGATCGTGTGGTCCGACATCCG<br>CACAGACGCCACGGTGGACCACATTCTGGCCAAGGTGCCCGAGAACAGCAA<br>GGACGCCCTGATGCCGCTGTGCGGGCTGCCCGATCACGCCCCTATATTTAGCG<br>CGCTCAAGATGCGCTGGCTCATCGACAACGTGCCCGCCGTGCGCAGGGCCAT<br>GCGGGACCGACGATGCCGGTTCGGGACGGTCGACTCGTGGCTCGTGTGGAA<br>CCTCACGGGAGGCAAGGACGGGGGGGCTCTTCATCACGGACGTGACCAACGC<br>CTCGCGCACCATGCTCATGAACATCGAGAGCCTCCAGTGGGACCCCGTGCTC<br>TGCCGCTACTTCAACATCTCTTCCAGCCTCCTCCCGGAGATCCGCAGCTCCTC<br>AGAGATTTACGGACACGTCACAGAGGAGCCCCTCAAAGGCGTGCCCATTTCC<br>GCCATTTTGGGCAACCAGCAATCATCCCTCATTGGAGAGAGGTGCTTCAACGT<br>CGGACAAGCCAAGGGCACTTACCGCAAGGGCTGCTTTCTCGTGTACAACACT<br>GGGTCGTCGCGAGTACATTCGACCCACGGATTAGAGACAACGGTGGCATATA<br>AACTGGGGAAAGATGCCCCAGCAGTGTATGCTCTGGAGGGCTCTGTGGCCGT<br>TGCAGGCAGCGCCATGAAGTGGCTTAAAGAAAACCTTAACGTCGTCAACGAA<br>GTCGGCGACATCGAAAGCGCTGCAGGGGAGGTGCCCTCGACTGGAGACGTG<br>TACTTTGTGCCTGCGTTTTCGGGGCTCTATGCCCCATACTGGAGGAAAGATGC<br>CAGAGGAATAATCTGTGGGCTGACCCAGTTTACAACCAAGCACCACATAATC<br>AGAGCGGCGCTGGAGTCGGTGTGTTTTCAGACCCGCGACATCCTCGAGTCAA<br>TGGCCAAAGACTGCAAGTGCCACCTGACCAAACTCCAAGTGGACGGCAAGA<br>TGACGGAGAACAAGTTGCTCATGCAGTTGCAGGCCGACATCTGCGGAATTCC<br>AGTCGAGCGAACCACGACTGATGTCAAGATGGATGCTCTTGGTGCGGCCATG<br>GCGGCCGGACAGGCGGAAGGCATCAATGTGTGGAATCTGGAGAAGGAGCCC<br>GACGGTCCGAGACCCACGAGGCGTTTATCCCACCACCAATCACGAAGAGCGC<br>GATGCACGATACACGAAATGGAAGATGGCCGTAGAAAAGGAGCTTGGGATGG<br>GCCACCACGAAAAAGTCGATTGCGATGACAGGTAAAATAACCGCTTCCGCTT<br>GAACCGACTCTGGAGAAAGTACAAAGTATTATCGTCACATGTGAAGCCGGAG<br>CCGTATCTGCCAAAAGCAAATAATTTTCATTTTAGCAGATATGAAGCTTCAATG<br>CAAATTAATCTGTGATTTTATGTGAAGTCACTTTCGGCAGTTGTGCCGTGTAA<br>ATATTTACAAGTATTGAGATCTGAGTATTGTTTTTAAGGTCGTTATTGTTGTTC<br>TTGTTCTTATTTAATGTCTCAAGAGACAAGACGCAGCTGTTTGTTGTGTGAGA<br>GAGATTTTACCAAAGTTATGTGTTGTTATATTTAAAGAAAGTTTATTAGTTTTGT<br>ATTTTTTGTGCATGAGTTGATGATGATTGTATTTGGGATTTGTTTGAACCCCAA<br>AATAAATCTGATATTATT |
| BGME17 | GATTAGCGTGGTCGCGGCCCTTCAAATAAATCAGTGGGCAGGTCATACTTTAT<br>ATATAAACAAAAAGAGATGTTTTTGTTAAACAGGCGAAAATAAAATTTGCCTA<br>ATTCCTAATAACTTATTACCAAAAATTAATTATTCCACTAATAATTTATACTAATT<br>TTACCTGCCCGGGCGGCCGCTCGAAATC |

| | |
|---|---|
| BGME18 | CTAAATTGTTTAATTATTTATTTTCTTCAACTTACTTTGAATATGAAAAAAATTG<br>AACAAGCATATAGCGTTAGGAGTTTACCGCCAATGTGTTCTTTTCAGAGAATC<br>GGCATTGTGAGAGTGTTGCAGTCGAGTGTCTGTGAAGCTGTTCTTCGTAATTT<br>CAGTTCAATAATGGCTCACCCACGAGTATTTTTCGACATGAGTGCCGATGGGC<br>AGCCTGTAGGAAGGATTGTGATGGAGCTCCGAAGTGATGTTGTACCAAAAAC<br>TGCAGAAAATTTCCGGGCTCTATGTACAGGAGAGAAAGGATTCGGTTATAAG<br>GGTAGCACCTTCCATCGTGTTATTCCCAACTTCATGTGCCAGGGTGGAGACTT<br>CACTAATCACAATGGCACTGGTGGAAAGTCAATTTATGGAACAAAGTTTGAG<br>GATGAGAACTTCCAGCTGAAGCACACTGGTCCAGGAATCCTTTCCATGGCCA<br>ATGCTGGACCCAATACTAACGGAAGCCAGTTCTTCATCACCACTGCAAAAAC<br>CAGTTGGTTGGACAATCGACATGTCGTTTTTGGATCTGTTGTCGAAGGAATGG<br>ACGTTGTGAAGAAGTTGGAGTCATTGGGATCGCAGAGTGGACAAAACAAAC<br>AAGAAGATCGCAGTAGTGGACTGTGGACAAATTTAAAAACTTAAGCTAAATG<br>TTTTCCTTTCCAAATTGCAGCATTAACATATTCCTGTTTTAAAAGCTAATTTCCA<br>TATGGATATAAAAAATATAAGTCACGACTGCAGTGTTTGGCGTGGATAGGCCA<br>AGAACCATCATTCCATACCTAGTTATTTTTTCTGTTTAGGTGGTGGTATGCCAA<br>AGAACTAAATCAACAACAACATGCAAAACCTCTCAATGAATAGTTCAACATG<br>TCCTAGCCATTATTATTATAATAATAATAAATAATAATTATAGGTAGTTTATATGGT<br>TATTGAAGAATCTTGCTGTAAATGTTTCTGATGTCGGAGCATTATCTAAATGTG<br>ATATTCATTTAGGAATTAGTCCATGCAGAAAAATAGGATTCCTTTATTTTTAAAC<br>AACTTGTAGTTAACATGTTTGCTGTCACAAGTCTTCAAAATATACCTTGCAGTC<br>CAATTTTGTTAAAATGTGCGTGCATATACTAATGTTGAAAGCAAAGTTTAACA<br>AAGCATTCCCAAGAATGAAGTACGTGCTTTTTTTTAAAACGAGGGTTTAATTT<br>TCTATTGCCTCTTTTGTTCAATTTTTTTCATATTCAAAGTAAGTTGAAGAAAAT<br>AAATAATTAAACAATTTAG |
| BGME19 | ATTTTAGTCGCCTATTTATTATAAAAGTGCAGTGATATAAAAAACAGTATACAG<br>CTATTTACAAATTCAGTCTTAAACTTAAAAATACTTTTTTCTTTACTGGCAATAT<br>CCATTGTAATTGCATGTGGCACCATCGGGACACTGGTAGTAACGTGCCTGAGC<br>TGGTACAGCCCTGCAGTTTCCTCCTATCGCAGTCTTGATGTAGCATGCATTACA<br>GCACTTGCATAATGCGCCGTCCGCTTTGAAAACGGTGCCATCTGGACAGTCA<br>GTTGCAGATTTTGGGGCACCGGTGCATTTTACTTGCAATTCTGTTTCACAAAC<br>TGTGGGGTCGTTACAGGTGGGAGGGAGTACAGCGTGTGAAACTGCAGCAAG<br>AAGAAGGAGACCCAAAGCTAGCTGGAACGTCTTCATCTTTTAATTAGTGGCTA<br>TTGTCC |

**Sequences of gene fragments obtained via SSH reverse operation (methoprene application as driver, acetone application as tester).**

| | |
|---|---|
| A1 | AGGCGAGTCGCATGCTCCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCC<br>CAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGG<br>CGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTC<br>CACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATG<br>AGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGG<br>GAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGG<br>CGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCT<br>CGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACG<br>GTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGG<br>CCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCAT<br>AGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGT<br>GGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTC<br>CCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCT<br>TTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTC<br>AGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCG<br>TTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCG<br>GTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCA<br>GAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTA<br>CGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTA<br>CCTTCGGAAAAGAGTTGGTAGCTCTTGATCCGGCAACAAACCACCGCTGGTA<br>GCGGTGGTTTTTTGTTGCAGCAGCAGATTACGCGCAGAAAAAAAGATCCT<br>CAAGAAGAATCCTTGATTCTTTTTCTTACGGGG |
| A2 | AAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTTTTTTT<br>TTTTTTTTTTTTTTTTTTTTTTGTTAATTTTTTGTTGAAAATGCAATTTCTTTTAT<br>CCCAAAAGCCTTTTACAGACTATGTCTAAACAAATAATTTTTAAACCAATAAA<br>ATATTGCACGGGATAACCCTTTAATAAATTTAATGGATAATT |
| A3 | AAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTTTTTTTTTTTTTTTTTTTT<br>TAAAAGTTTACCCTACAGTAAAAATGTAATAATTTTATTATTTCACAATGTATGT<br>ATATTTCACAATAAAAATAAATATACGTTATATTACGGGGAAAAATTTAAAAAT<br>TTTCTTGTATACAAAAATAAACCCCGGCCGCGACCCCCCTAATCACTAGG |