



Pharmacological analysis on mechanism that modulates developmental rate, body size, activity, circadian rhythms and metabolism in *Gryllus varius*[Gryllidae: Orthoptera]

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Doctoral Dissertation

博士論文

**Pharmacological analysis on mechanism that modulates
developmental rate, body size, activity, circadian rhythms and
metabolism in *Gryllus varius* [Gryllidae: Orthoptera]**

**コオロギ *Gryllus varius* における発育速度、体サイズ、
活動性、概日リズムおよび代謝調節機構の薬理学的分析**

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**I dedicate this thesis to my dearest parents, for their
emotional support and selfless love.**

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Chapter 1

General introduction

Crickets as a simple model for sleep mechanism like other hemimetabola undergo egg, nymph and adult stages for post-embryonic development, molting several times. The number of molts or the number of instars ranges from 5-13 in *Gryllus sp.* (Huber, 1989). According to our observation, the number of molts in the cricket, *Gryllus varius* (Gryllidae: Orthoptera) is 7 under LD 12:12 and 5 under LD 16:8 with an average of 92 days and 75 days at 30°C to reach adulthood, respectively. The females of this species laid eggs continually in our culture conditions.

Most crickets have two pairs of wings, including male forewings specialized for calling and hind wings specialized for flying. There is alary dimorphism in this species, which produces both brachypters and macropters.

Most crickets are omnivorous, and the food range varies among species. Development during the whole life of crickets depends on photoperiod and temperature (Tanaka, 1979, 1984; Forrest, 1987). The activity of crickets is also modulated by the photoperiod, and shows daily rhythms generated by endogenous oscillator, which are entrained by the light cycles (Saunders, 2002). Many crickets are nocturnal (Loher 1972; Nielsen and Dreisig 1970; Nowosielski and Patton 1963), which shows that their activity (walking, feeding, and stridulation) occurs mostly in the scotophase (FaBold, 2010). Besides, metabolic study shows that metabolic rate is

higher at walking or singing than at rest in crickets (Huber et al., 1989).

Circadian oscillation is the endogenous, entrainable oscillation of the biological process. This mechanism is usually driven by the environmental cycle. The external cycles, such as light/dark cycle and temperature cycles, entrain circadian rhythms. The circadian oscillator responds to the stimulation of day-night cycle by phase shifts of certain magnitude at fixed phase points when steady-state entrainment is attained. At the molecular level, the circadian clock genes modulate the circadian rhythm in most organisms (Bargiello et al., 1984; Shearman et al., 2000).

Melatonin (*N*-acetyl-5-methoxytryptamine) is a naturally occurring compound found in animals, plants, and microbes (Caniato, 2003; Paredes, 2009). In animals, melatonin secretion varies in a daily cycle. Melatonin serves as the entraining agent of the circadian rhythms of several biological functions (Altun, 2007). Wetterberg et al (1987) demonstrated that melatonin is present in the human brain and cerebrospinal fluid peaks at night of the day-night cycle. It is believed that melatonin is masking the light, and cephalic levels of melatonin appear to be higher during the scotophase than during the photophase. Melatonin synthesis is probably modulated by photoperiod or the light suppresses it. However in the giant freshwater prawn, melatonin appears to be higher during daytime than during night time (Withyachumnarnkul, 1992).

It has been widely believed that melatonin is produced in the pineal gland (Reiter, 1991), but now, it well proves that melatonin is present and synthesized in other organs such as the retina and digestive tract, sensitive to environmental changes.

(Vivien-Roels and Pevet, 1983). Melatonin in invertebrates was first found in the compound eyes of the locust, *Locusta migratoria* by GC-MS (Vivien-Roels et al., 1984).

As far as we know, the retina is a photoreceptor perceiving light. Upon perception of light at the retina, the retinobypothalamic tract conveys glutamatergic signal to the suprachiasmatic nuclei that contains the circadian pacemaker (CPM). The CPM sends neural tracts to the superior cervical ganglion that sends adrenergic signal to the pinealocytes. Further evidence has shown that the circadian pacemaker is also in the optic lobes (Page, 1982, 1985). Since melatonin is present and rhythmically synthesized in the optic lobe of the cockroach (Vivien-Roels and Pevet, 1993), it could regulate the circadian rhythm by affecting the oscillators and/or pacemaker. It has been convinced that melatonin depressed the amplitude of rhythmic contractions via cyclic guanosine monophosphate (cGMP) (Ancil et al., 1991).

On the other hand, melatonin regulates the sleep–wake cycle, causes drowsiness and reduced the body temperature. It has been used to treat circadian rhythm sleep disorders, such as delayed sleep phase syndrome (DSPS), non-24-hour sleep-wake syndrome where circadian rhythms are not entrained to the environmental cycle, jet lag and the problems of people who work on rotating or night shifts (Buscemi, 2004).

Melatonin is synthesized in the pinealocyte by arylalkylamine N-acetyltransferase (aaNAT) that is degraded even by a brief light. It is a potent terminal antioxidant that can penetrate most biological membranes (Pohanka, 2011) and the blood–brain barrier

(Hardeland, 2005; Reiter, 2010). Application of melatonin may protect against oxidative damage and prolong aging caused by the neurodegenerative process (Pohanka, 2011). Oral administration of low dose of melatonin reduced the oxidative damage caused by aging and delayed the inflammatory process, which in turn increased the longevity of the mice (Acuna-Castroviejo, 2001).

Because of its interaction with the immune system (Carrillo-Vico, 2005), melatonin might be useful for fighting infectious disease including viral (Maestroni, 2001), such as HIV, and bacterial infections, and potentially in the treatment of cancer (Maestroni, 1999). Studies in rats suggest that melatonin may be effective for treating Alzheimer's disease (Wang, 2005). On the other hand, melatonin was thought to have a very low toxicity in rats (Jahnke, 1999), and Wiechmann (2008) reported that it is toxic to photoreceptor cells in rats' retinas.

There are three melatonin receptors, MT1, MT2, and MT3 in human subjects that are G-protein coupled receptors (Reppert, 1997). MT1 and MT2 receptors are linked to activation of multiple signaling pathways, with inhibition of cAMP formation through pertussis toxin sensitive G-proteins (Dubocovich, 2003). The Study also shows that melatonin inhibited the calcium-dependent release of ³H-dopamine from rabbit retina (Margarita, 1983). Activation of MT2 in the rat SCN appears to phase shift in circadian rhythms of neuronal firing (Hunt et al., 2001). The activation of MT1 and/or MT2 may regulate the melatonin function to modulate the amplitude of the circadian signal and the phase shift of circadian rhythms of neuronal firing, and

then shift the sleep timing (Dubocovich, 2005; Hunt et al., 2001; Liu et al., 1997).

Caffeine (1,3,7-trimethylxanthine) is present in a number dietary sources consumed worldwide, such as tea, coffee, cocoa beverages, and other soft drinks. It is found in the seeds, leaves, and fruit of some plants, where it acts as a natural pesticide that paralyzes and kills certain insects feeding on the plants. In humans, caffeine acts as a stimulant of central nervous system, temporarily warding off drowsiness and restoring alertness. It is completely absorbed in animals (Arnaud, 1976, 1985) while it is also toxic to animals above certain concentrations at the same time (Eichler, 1976; Itoyama and Bicudo, 1992; Laranja et al, 2003; Nikitin et al., 2008).

Caffeine effects are distinct at different concentrations and also varies among different individuals. It depressed the locomotor activity at high doses while the low does increased it (Daly, 1993; Yacoubi et al., 2000). It lengthened circadian period and delayed the phase of circadian clock (Oike et al., 2011). Caffeine affected the clock genes expression and it is tissue dependent (Sherman et al., 2011). In addition, caffeine depressed melatonin in humans at night (Wright et al., 1997). Similarly to melatonin, certain concentrations of caffeine also increase the longevity (Lublin et al., 2008; Sutphin et al., 2012; Wanke et al., 2008), though it reduces the longevity of insects, and toxic to them at other concentrations (Itoyama and Bicudo, 1992; Laranja et al, 2003; Ninitin et al., 2008).

Caffeine can pass all biological membranes and is distributed to the whole body of animals. Effects of caffeine on sleep are very different among individuals, as

metabolic rate or the sensitivity to caffeine is variable among different individuals (Fredholm, 1999). It can increase the rates of cerebral energy metabolism in the rat, and the locomotor activity but delay the onset of sleep. However, some studies have shown that caffeine does not stimulate the activity all the time. The effect on the movement is dose-dependent (Daly, 1993; Yacoubi et al., 2000). In humans, there are also some similar effects (Wardle et al., 2012). The main productions of metabolites of caffeine are paraxanthine and theophylline, and the former can stimulate the lipolysis process which releases glycerol and fatty acids into the blood as a fuel for the muscles while the latter increases heart rate (Dews, 1984).

In humans, coffee consumption results in a lower overall risk of cancer, some age-related diseases such as dementia, Alzheimer's, Parkinson's disease, and type 2 diabetes, and in the improved learning and memory (Ascherio et al., 2001; Marques, 2011; Nehlig, 2010; Nkondjock, 2009; van Dam, 2008). The seniors that habitually consume caffeine have reduced mortality and improved life span (Paganini-Hill et al., 2007; Santoc et al., 2010; Sutphin et al., 2012).

At the molecular level, caffeine blocks adenosine receptor A1, A2 and A3 directly (Fredholm, 1980, 1995) and then arouses a series of stimulations at molecular and cell levels. It increases cAMP formation and transmitter releases (Chavez-Noriega and Stevens, 1994; Bode and Dong, 2007), speeds up the central neurons firing, stimulates dopamine receptors activation (Ferre et al., 1997; Fredholm, 1999), increases so-called immediate early gene (IEGs) (Johansson et al., 1994; Svenningsson et al.,

1995), blocks GABA-A receptors (Lopez et al., 1989; Fredholm, 1999), enhances synaptic NMDA receptor activities (Powell and Holtzman, 1998; Fredholm, 1999), activates ryanodine receptors (McPherson et al., 1991), and reduces the threshold of Ca^{2+} release pathway (Friel, 1992; Fredholm, 1999). It is widely accepted as an antagonist of adenosine, which is well known to reduce neural activity level. This evidence explains why caffeine causes anxiety, insomnia, rapid heart and respiration rate (Fredholm, 1999).

Besides the above study, the progress in the behavioral study with crickets, functions of melatonin and caffeine, and the molecular mechanisms of both medicines prompted us to study the effects of melatonin and caffeine on the behavior of the cricket, *Gryllus vavivus*, and other physiological phenomena. Melatonin is well-studied for the effect on circadian rhythm and caffeine with the effect on locomotor activity, respectively. Most knowledge came from mammals, especially rats and mice, while studies of two medicines on the activity in insects are rare. Commonly, it is believed that melatonin and caffeine have the opposite functions in such that the former is a stimulant whereas the latter is a sedative. On the other hand, recent studies show that the functions of melatonin and caffeine are not always stable, like some other drugs, they have both advantages and disadvantages to animals which depend on the doses. In our study, we chose three relatively low concentrations, 100, 200, 400 $\mu\text{g/ml}$ of both medicines to test their effects in the crickets. The results indicate that melatonin affected physiology conversely between at nymph and adult stages, while caffeine affected nymphs conversely between different photoperiods. Both of them shift the

activity phase and rest phase simultaneously, and even change the growth and metabolic rate at the same time. Finally, they affected the circadian rhythm in adults particularly.

Chapter 2

Effects of melatonin and caffeine on behavior, metabolic rate, growth duration and body size in the cricket

2.1 Abstract

Melatonin affected nymphs under LD 12:12 and LD 16:8 with the same tendency: increased the activity, decreased the rest time and growth duration, and increased the metabolic rate. The fraction of night activity decreased and that of night rest increased. The body size increased slightly as well. It seems that melatonin took functions independently of light, and the effects under LD 12:12 were more significant than under LD 16:8. Melatonin affected males and females with the same tendency as well. But the effects were opposite to that in nymphs. Melatonin extended the life span of adult stage, decreased the metabolic rate and activity. Males were sensitive to melatonin than females.

The effects of caffeine in nymphs under LD 12:12 and LD 16:8 were converse. It increased the activity under LD 16:8 whereas decreasing that under LD 12:12. And relatively, the rest decreased, metabolic rate increased, and growth duration decreased under LD 16:8 while the characters were affected conversely under LD 12:12. It seem that caffeine took actions dependently of light, and the differences of effects under these two photoperiods were significantly. For adults, caffeine had the same effect tendency with melatonin, which decreased the activity, increased the rest, decreased

the metabolic rate, and than prolonged the life span of two sexes. The fraction of night activity increased while that of night rest decreased. The significance of differences between two medicines' effects were not obvious but in nymphs under LD 12:12. The Characters used in the study showed some relationship that the crickets activated less but grew longer if they fed less and the body size also became smaller.

Key words: melatonin, caffeine, activity, rest, growth duration, metabolic rate, body size, fraction of night activity, fraction of night rest

2.2 Introduction

There is the compact relationship between metabolic rate, behavior and growth. High metabolic rate induces high activity and fast development, and at the same time, the body size or the weight will also be influenced by the metabolism (Bertalanffy, 1951; Davidowitz and Nhhoutt, 2004; Davidowitz et al., 2004).

Melatonin is well known as a regulator of sleep-waking cycle, which takes functions through MT1, MT2 receptors (Dubocovich, 1995; Dubocovich et al., 1997) to inhibit the release of dopamine from retina (Dubocovich, 1995). The secretion of melatonin mainly occurs at night (Dubocovich et al., 1998; Vanhoutte et al., 1996) and in the circadian rhythmic way in the brain. Many studies have proved that melatonin decreased locomotor activity and improve the sleep in mammals and insects (Azpeleta, 2010; Murakami, 2001) and prolongs their life span (Acuña-Castroviejo, 2001; Bonilla et al., 2002; Pohanka, 2011). Recent study shows that the rats lose weight after melatonin administration, which is due to its effect to

decrease food and water intake and metabolism (Ahmed, 2011).

Caffeine is well known for its stimulation on activity and mood in animals. It makes humans excited, and even anxious (Nehlig, 1992). At the same time, caffeine is a dose-dependent stimulant and affects in an individual-dependant form (Fredholm et al., 1999). Some studies have proved that it decreases the activity in rats, mice (Daly, 1993; Yacoubi et al., 2000). The most acceptable molecular mechanism of caffeine in the brain is that caffeine mainly combines A1 and A2 adenosine receptors (Fredholm, 1955, 1980), to induce other physiological responses at molecular level (Fredholm et al., 1999). Caffeine also is reported to prolong the life span of animals, and is also dose-dependant (Itoyama et al., 1992; Lublin et al., 2008; Nikitin et al., 2008; Wanke et al., 2008; Sutphin et al., 2012; Yusaf, 2012) and the explanation of age increase still needs to investigate.

This chapter investigated the effects of melatonin and caffeine on behaviors, metabolic rate, growth duration and body size of the cricket, *Gryllus vavivus*, respectively. We tested its nymph and adult stages. In order to compare the functions, we tested the same concentration gradient for both medicines, and the result showed that melatonin and caffeine were not conversely all the time. Both of them were dose-dependant, and had the relationship with different individuals, photoperiods and temperatures.

2.3 Material and methods

2.3.1 Insect

The Mexico cricket, *Gryllus vavii* was collected from Teotihuacan, Mexico (19°28'N 99°09'W) and reared in incubators at 27.5°C, LD 16:8 in egg, and 30°C, LD 16:8 and LD 12:12 in nymphs, and 20°C, LD 16:8 in adult stage. They were fed with an artificial diet mouse food (MF, ORIENTAL YEAST Co. Ltd.) and water. The nymphs under LD16:8 and LD 12:12, brachypter and virgin males and females were studied to simplify the experiment though the species shows alary dimorphism.

2.3.2 Medicines

Melatonin and caffeine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The same three concentrations of both medicines were prepared, 100, 200, and 400 µg/ml, and tested their effects by oral administration. Melatonin was dissolved firstly in ethanol and then in distilled water to desired concentrations. Caffeine was dissolved in distilled water to the desired concentrations directly and supplied to the adult crickets. Water was used as control. Medicines were put into the foil-covered bottles, and given to nymphs from hatch and to adults from eclosion. Medicines were renewed every 5 days.

2.3.3 Behaviors Study

Video recording was used to study the behaviors of the crickets, including rest and activity. The behavior was marked as rest when the crickets stood still without moving any part of the body. Food and water intake, walking, and any movement of the body were defined as activity. These behaviors were recorded every 5 min, and analyzed for 12 h at hourly intervals, which was similar to that described by Nishio (2009).

One individual was monitored by the camera HDR-CX560 (Sony Co. Ltd., Tokyo Japan) in one chamber made of transparent plastic plate (100×65×28mm), which contained solid mouse food (MF, ORIENTAL YEAST Co. Ltd.) and water (Fig. 1). The animation was preserved in HDD recorder, VARDIA[®] RD-S600 (TOSHIBA Co. Ltd., Tokyo Japan). The chambers were put into the incubator under the same rearing conditions described above for nymphs and adult, respectively (Fig. 2).

The recording started from around 40 days after hatch for nymph and around 20 days after eclosion for adults. Each individual was recorded for a day on nymph stage, and also a day on adult stage.

2.3.4 Body sizes measurement

To study the effects of medicines on growth of nymphs, body sizes on adult stage were measured after the eclosion for each tested individuals. The pronotum width and metathoracic tibia length were measured for males only, and the measured males were emerged from nymphs reared under LD 16:8 and LD 12:12, respectively.

2.3.5 Growth duration test

To study the effects of medicines on growth rate, the growth duration of nymph and adult stages were tested. It contains the period of nymph stage from hatch to eclosion and the period of adult from eclosion to death.

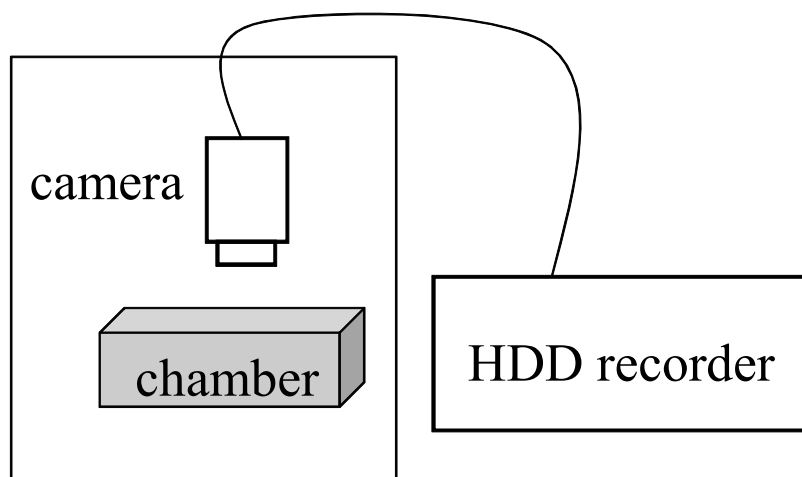


Fig.1 The video recording system used in this study, which was described in the text. One individual was put in a plastic chamber, and behaviors inside were recorded for one day.

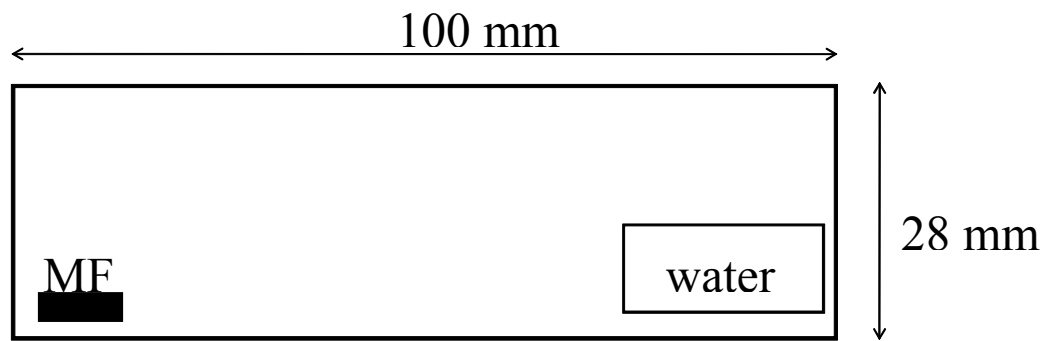


Fig. 2 The test chamber for video observation from above. The water bottle was wrapped with foil, containing melatonin or caffeine solutions of designated concentration. MF: mouse food.

2.3.6 Feces weight

To measure the metabolic rate with or without drinking medicines, the feces of individuals are weighed after the video recording. The value is the volume of the feces during the one-day recording. We propose that the metabolic rate is proportional to the feces volume, which means that more metabolic rate leads more feces is released. The food will become powder bitten by the crickets, which is inaccurate to be weighed. This is alternative reason we chose feces not the leftover food.

2.3.7 Data analysis and statistics

The behavior characters included the time of the two kinds of behavior (rest, activity), and the fraction of the night behavior (N/T of activity and N/T of rest). These characters were analyzed for nymphs and adults respectively after drinking caffeine and melatonin based on the cumulative data from the video recording. For nymphs, one-way ANOVA was performed to determine the effect of the medicines under LD 16:8 and LD 12:12, respectively, and *post hoc* analysis were realized with Fisher's LSD where appropriate. To compare the effects of the medicines in these two different photoperiods, two-way ANOVA was subsequently performed (photoperiod $\times$ dose). For adults, meanwhile, one-way ANOVA was performed to determine the effect of the medicines in males and females, respectively, and *post hoc* analysis was realized with Fisher's LSD where appropriate. To compare the effects of the medicines in sexes, two-way ANOVA was subsequently performed (sex $\times$ dose). Values were expressed as means $\pm$ SD, $p < 0.05$ was considered the level of

significant difference based on *post hoc* analysis (Fisher's LSD).

2.4 Results

2.4.1 Effects of melatonin on nymphs under LD 16:8 and LD 12:12

a) Effects of melatonin on nymphs under LD 12:12

Fig. 3 shows an example of the record of behaviors from a nymph under LD 12:12. After drinking melatonin, the activity increased significantly while the rest time decreased. Both effects were significant in 100 µg/ml melatonin-treated group (LSD, $p < 0.05$, Fig. 4 and Fig. 5). Growth duration of nymph stage in LD 12:12 shortened and significantly in 400 µg/ml group (LSD, $p < 0.05$, Fig. 8). The feces increased as the concentration of melatonin increased. 400 µg/ml group was different significantly, but 200 µg/ml group was different most significantly and fed most, (LSD, $p < 0.01$ in 200 µg/ml group, $p < 0.05$ in 400 µg/ml group, Fig. 6). Night rest fraction increased, especially significantly in 200 and 400 µg/ml groups (LSD, $p < 0.05$, Fig. 11), while the night activity fraction decreased, and the fraction in 200 and 400 µg/ml group decreased significantly (LSD, $p < 0.05$, Fig. 9). Moreover, the pronotum width and metathoracic tibia length decreased slightly in melatonin-treated groups compared with control, though not significantly (Fig. 13 and Fig. 14).

Melatonin in nymphs under LD 12:12 changed activity and rest time significantly, which showed that it increased the activity but decreased the rest period at the same time. It also induced that more than half of the rest fraction occurred in the dark, while shifting activity to the light significantly.

b) Effects of melatonin on nymphs under LD 16:8

After drinking melatonin, the activity increased but the rest time decreased, both were different significantly in 100 µg/ml group (LSD, $p < 0.05$, Fig. 4 and Fig. 5), while growth duration of the nymph stage decreased as the concentration increased, especially decreased significantly with 200 and 400 µg/ml treatment (LSD, $p < 0.05$ in 200 µg/ml group, $p < 0.01$ in 400 µg/ml group, Fig. 8). Meanwhile, the feces increased as the concentration of melatonin increased and significantly after drinking 400 µg/ml melatonin (LSD, $p < 0.05$, Fig. 7). The fraction of night rest increased significantly in 200 and 400 µg/ml group (LSD, $p < 0.05$, Fig. 12). In contrast, the night activity fraction decreased as the concentration increased and significantly in 400 µg/ml group, except 100 µg/ml treatment, which increased the fraction of night activity slightly (LSD, $p < 0.05$, Fig. 10). Moreover, pronotum width and length of metathoracic tibia decreased slightly compared with control (Fig. 13 and Fig. 14).

Under LD 16:8, similar to that under LD 12:12, melatonin changed the distribution of rest and activity during one day significantly, as well as the ratios of these two behaviors. In control, less than half of rest occurred in the night while more than half moved to the night in melatonin treatments (200 and 400 µg/ml, Fig. 12). On the other hand, activity became more centered to the light phase as activity decreased in the dark. Combined the increase on feces and decrease on growth duration under both LD 12:12 and LD 16:8, melatonin may stimulate nymphs to feed more to grow more rapidly.

c) Comparison between LD 12:12 and LD 16:8

Under two different photoperiods, melatonin affected behavior, growth duration and feces by the same trend, which increased activity level and diet, decreased rest time, body sizes and growth duration of nymph stage. On the other hand, the parameters analyzed here were significantly different under two photoperiods. In details, the stock under LD 16: 8 acted more, rest less, feed more, and growth more rapidly, compared with stock under LD 12:12. Among them, the differences were significant in activity, rest time, and the body sizes ($p < 0.01$, two-way ANOVA). Besides, according the statistical analysis, melatonin affected crickets more obviously under LD 12:12 than under LD 16:8.

2.4.2 Effects of caffeine on nymphs under LD 16:8 and LD 12:12

a) Effects of caffeine on nymphs under LD 12:12

According to the result, the activity decreased except for that of 400 $\mu\text{g/ml}$ caffeine-treated group, while the rest time increased after drinking caffeine, though not significantly (Fig. 15 and Fig. 16). The growth duration of nymph stage in LD 12:12 prolonged in caffeine groups and maximum in 200 $\mu\text{g/ml}$ group (Fig. 18). The feces decreased as the concentration of caffeine increased, except that in 400 $\mu\text{g/ml}$ group (Fig. 17). The night rest fraction increased, especially significantly in 200 $\mu\text{g/ml}$ groups (LSD, $p < 0.05$, Fig. 19), while the fraction of night activity decreased, and the fraction in 200 $\mu\text{g/ml}$ group decreased most (LSD, $p < 0.05$, Fig. 21). The pronotum width and length of metathoracic tibia increased slightly (Fig. 23 and Fig. 24).

Caffeine changed the rest and activity period of nymphs in LD 12:12, which

showed that it decreased the activity while increased the rest time. It changed the distribution of activity and rest significantly during one day. The increased age and decreased activity and feces indicate that caffeine may delay the development of nymphs by restricting diet and lower the activity level to save the energy and then the body size became bigger may be because they grew more slowly than control.

b) Effects of caffeine on nymphs under LD 16:8

After drinking caffeine, the rest time decreased and significantly in 100 µg/ml group (LSD, $p < 0.05$, Fig. 16) while activity increased compared with that in control, though not significantly (Fig. 15). The growth duration of the nymph stage decreased as the concentrations increased (Fig. 18). Meanwhile, the feces increased as the concentration of melatonin increased significantly after drinking caffeine (LSD, $p < 0.05$, Fig. 17). The fraction of night rest decreased, (Fig. 20). In contrast, the fraction of night activity increased after drinking caffeine (Fig. 22). The pronotum width and metathoracic tibia length decreased slightly after drinking caffeine (Fig. 23 and Fig. 24).

Under LD 16:8, caffeine changed the distribution of rest and activity during one day slightly, and the ratios of these two behaviors significantly during one day. It induced nymphs to activate more in the dark while rest more in the light, which has the opposite effects to melatonin described in **study 1** (Fig. 12 vs. Fig. 20 and Fig. 10 vs. Fig 22). Combined the increase in feces and activity time, decrease in growth duration and shorted body size, caffeine may stimulate nymphs to become excited and feed more, which may lead to grow faster and have a smaller body size in LD 16:8.

c) Comparison between LD 12:12 and LD 16:8

Different from melatonin, caffeine affected crickets oppositely under two different photoperiods. Under 12:12, it depressed the activity of crickets and prolonged the development because of increased growth duration. But it stimulated the activity and speeded up the development with decreased growth duration under LD 16:8. Besides, the night activity fraction decreased under LD 12:12 while that under LD 16:8 increased. And change of the fraction of night rest had the converse result. According to the statistical analysis, the opposite effects of caffeine under LD 12:12 and LD 16:8 were significantly different ($p < 0.05$, two-way ANOVA).

2.4.3 Effects of melatonin on males and females

a) Effects of melatonin on males

For males, after drinking melatonin, the activity decreased while the rest time increased, and significantly in 100 $\mu\text{g/ml}$ group (LSD, $p < 0.05$, Fig. 25 and Fig. 26). The growth duration of adult stage increased and maximized in 200 $\mu\text{g/ml}$ group (approximately 10 days, Fig. 28). Meanwhile, the feces decreased as the concentration of melatonin increased and significantly after drinking 400 $\mu\text{g/ml}$ caffeine (LSD, $p < 0.05$, Fig. 27). The night activity fraction increased after drinking melatonin, and was highest in 100 $\mu\text{g/ml}$ group (Fig. 29). In contrast, the night rest fraction decreased, and was lowest in 100 $\mu\text{g/ml}$ group, though not significantly (Fig. 30).

In males, melatonin decreased the activity but increased the rest time, and the fraction of night activity increased as well while that of rest decreased. The decreased activity, feces and increased growth duration of adult stage in males indicate that

melatonin prolonged the life span in males with these three concentrations by restricting diet and depressing the activity.

b) Effects of melatonin on females

In females, melatonin induced the similar effects on behavior, feces and the growth, the activity decreased and rest increased (Fig. 25 and Fig. 26), growth duration of adult stage increased and has the maximum in 200 µg/ml group (increased approximately 20 days, Fig. 28). Feces decreased as the concentration of melatonin increased and significantly decreased in 400 µg/ml group (LSD, $p < 0.05$, Fig. 27). The night activity fraction increased, while the night rest fraction decreased (Fig. 29 and Fig. 30).

a) Comparison of melatonin effects between males and females

Melatonin affected males and females by the same trend. It depressed the activity and increased rest time, and prolonged growth duration of adult stage, while feces decreased in both sexes. Melatonin changed the behavior in males significantly but not in females. According to the statistical analysis, the effects of melatonin were significantly different on activity and rest in both sexes ($p < 0.05$, two-way ANOVA). Males were more sensitive to melatonin than females.

2.4.4 Effects of caffeine in males and females

a) Effects of caffeine in males

In males, caffeine decreased the activity but increased rest, obviously most in 100 µg/ml group (Fig. 31 and Fig. 32). The feces decreased after drinking caffeine, especially in 200 µg/ml group (Fig. 33). In contrast, growth duration of adult stage

increased, and most in 400 µg/ml group (approximately 20 days, Fig. 34). The fraction of night activity increased while that of night rest decreased after drinking caffeine (Fig. 35 and Fig. 36).

The decreased activity, feces and increased growth duration of adult stage in males indicate that caffeine may prolong the life span in males by restricting diet and depressing the activity with these three concentrations. Besides, caffeine modulated the distribution of activity and rest between the light and dark period, activity moved towards the dark phase while the rest moved towards the light phase.

b) Effects of caffeine in females

In females, caffeine had the similar effects on behavior, feces and life span. The increased behavior was most obvious in 100 µg/ml group (Fig. 31), as well as the decreased feces and increased growth duration (Fig. 32). The fraction of night activity increased slightly but that of night rest decreased significantly in 200µg/ml caffeine-treated group (LSD, $p < 0.05$, Fig. 35 and Fig. 36).

The decreased activity, feces and increased growth duration of adult stage in females indicate again that caffeine prolonged the life span by restricting diet and depressing the activity.

With the same trend of effects of caffeine in males, there is necessary to analyze the significant difference between sexes.

c) Comparison of caffeine effects between males and females

Caffeine affected males and females by the same trend. It depressed the activity and increased the rest time, and prolonged growth duration of both sexes, while the

feces decreased in them. Caffeine with these three concentrations changed the behavior in both sexes, though not significantly in all parameters. And two-way ANOVA showed that there was no significant difference between sexes, but the parameters changed more obviously in males than in females (Fig. 31, Fig. 32, Fig. 33, Fig. 34, and Fig. 35) in LD 16:8, at 20°C.

2.4.5 Comparison of effects between melatonin and caffeine in nymphs

Under LD 12:12, melatonin increased the activity and decreased the rest whereas caffeine decreased the activity and increased the rest (Fig. 4 vs. Fig. 15; Fig. 5 vs. Fig. 16), and the differences between two medicines were significant ($p < 0.05$, two-way ANOVA). The growth duration of nymph stage was shortened by melatonin but prolonged by caffeine (Fig. 8 vs. Fig. 18), and the differences also were significant ($p < 0.05$, two-way ANOVA). The feces increased with melatonin treatment but decreased with caffeine treatment, though not significantly different (Fig. 6 vs. Fig. 17). The fraction of night activity decreased and that of night rest increased by melatonin while caffeine affected it conversely, though not significantly different (Fig. 9 vs. Fig. 21; Fig. 11 vs. Fig. 19). Besides, melatonin shortened the body size but caffeine lengthened it slightly (Fig. 13 vs. Fig. 23; Fig. 14 vs. Fig. 24). Moreover, nymphs were more sensitive to melatonin than to caffeine under LD 12:12.

Under LD 16:8, melatonin and caffeine affected nymphs in a similar way. Both of them stimulate the activity and depressed the rest, increased food and water intake and then shortened growth duration of nymph stage. At the same time, there was obvious difference of effects on feces from these two medicines ($p < 0.05$, two-way ANOVA).

On the other hand, the fraction of night activity decreased and that of night rest increased in melatonin drinking groups whereas caffeine affected adversely. Finally, in both medicines, the most effective concentrations were distant. In melatonin, 400 µg/ml concentration while 100 µg/ml concentration in caffeine, affected most obviously.

2.4.6 Comparison of effects between melatonin and caffeine in adults

For both sexes, the effects of melatonin and caffeine showed the similar trend. They suppressed the activity, increased the rest, restricted diet and prolonged growth duration of both sexes. The fraction of night activity increased but the fraction of night rest decreased. And there was no significant difference between the effects of these two medicines. Moreover, males are more sensitive to both medicines than females, which means the behaviors changed more in males than in females (Fig. 25, Fig.26, Fig. 31, Fig. 32).

2.5 Discussion

We reared the nymphs in two different photoperiods, and they showed different behavior levels and growth paces. Under LD 16:8, the nymphs grew more rapidly and had the longer activity and shorter rest. At the same time we got more feces which reflected that the stock fed more. The body size seems also having the relationships with the growth rate, because the body size was smaller under LD 16:8 than that under LD 12:12. Photoperiods affect the development of nymphs in insects (Beck, 1975), and the cricket, *Velarifictonus ornatus* also developed faster under LD 16:8

than under LD 12:12 (Zhao et al., 2008). Body size is smaller at the higher growth rate by reducing the mass that rats can accumulate (Davidowitz and Nijhout, 2004). At the same time, the metabolic rate is relevant to the body size, and the metabolic rate is higher in smaller animals than in bigger ones (Kleiber, 1947). Reduction of food intake has been shown to slow ageing and extend life span (Maroso, 2005). All these reports are consistent to our results. In our study, the stock with low growth rate under LD 12:12 activated less but rest longer than that with high growth rate under LD 16:8. We speculate that activity level has the relationship with the growth duration. Low metabolic rate may induce the depressed activity, save the energy and then slow the growth rate of nymph stage. In a word, there is a chain that connects metabolic rate, activity level, growth rate, and body size together. One of them changes may induce other characters to have some responses. This is the base to study the effects of melatonin and caffeine on physiological functions of our crickets.

Melatonin and caffeine affected nymphs under LD 12:12 and LD 16:8 and the series of effects conformed to the chain of the characters studied here. In details, melatonin increased the activity level, decreased the rest, and increased the growth rate and the feed, which means the metabolic rate increased as well. The decreased body size also reflected the physiological change after drinking melatonin. On the other hand, it affected adult in the opposite way, which caused activity decreased, rest increased, lengthened the growth duration and the metabolic rate decreased.

Caffeine showed the same trend of effects as melatonin in adults. But in nymphs, it showed bilateral effects under different photoperiods. In details, it increased the

activity under LD 16:8, but decreased it under LD 12:12 conversely and other characters changed appropriately. And the differences were significant between two photoperiods.

Melatonin stimulated the activity in nymphs while depressed it in adults, which indicates that melatonin take function depends on different stages of the insects. The same trends in nymphs under different photoperiods or in different sexes indicate that melatonin is masking the effect of the modulation of the light. It may function independently of light. On the other hand, different trends in nymphs under two photoperiods after drinking caffeine indicates that caffeine functions dependently of light, it may cooperate with the light to take functions or the light affects the modulation of caffeine.

Gaffori (1985) studied that melatonin suppressed the locomotor activity and feeding in adult rats, which suggested that melatonin induces low activity by antagonizing serotonin. Another explanation is that melatonin decreased neuronal firing in the SCN through M1 and M2 receptors (Margarita, 2007).

The secretion of melatonin is in a circadian rhythmic manner and mainly confined to the dark (Axelrod, 1974). It can shift the circadian rhythms of neuronal firing, which may affect rest timing (Kilduff, 1998; Kunz, 2004).

The study shows that melatonin increases the metabolic rate of rats to lose weight, though the feeding does not change significantly (Wolden-Hanson, 2000). In our study, melatonin also increased the metabolic rate in nymphs but they fed more.

The dilatory intake of melatonin has been shown to extend the life span of fruit

fly, *Drosophila melanogaster* significantly (Bonilla et al., 2002). They suggested that melatonin prevents oxidative damage to the fly tissues and slow down the process of aging. Melatonin also delays the children growth and has the relationship with growth hormone (Munoz-Hoyos, 2001).

The general views consider that caffeine stimulates activity and wakefulness of rats, mice and our humans and restores the alertness (Waldeck, 1975; Bolton and Sanford 1981; Nehlig et al., 1992; Daly, 1993; Marcelo et al., 2011). This is because caffeine blocks adenosine receptors and then increases cAMP formation and dopaminergic transmission to fire the neurons (Fredholm et al., 1999), this can explain why the activity increased in caffeine-treated nymphs in LD 16:8. In contrast, caffeine has been show to decrease the activity of dopaminergic neurons (Stoner et al., 1988) and stimulate the low behaviors under moderate concentrations (Svenningsson et al., 1995, 1997). The universal law is low dose caffeine stimulates the locomotor behavior while the high does depresses the motor (Boissier and Simon, 1965; Waldeck, 1975; Logan et al., 1986; Nikodijevic et al., 1993; Yacoubi et al., 2000; Marin et al., 2011). Studies of caffeine effects on animals indicate that caffeine has the biphasic effects on animals which depend on the does of caffeine and the individual differences. On the molecular level, caffeine is considered as A₁ and A_{2A} receptor antagonist (Fredholm, 1980, 1995). On mice, the stimulant effect of low doses of caffeine is mediated by A_{2A} receptor blockade while the depressant effect seen at higher dose explained by A₁ receptor blockade (Yacoubi et al., 2011). On the other hand, the direct injection of an adenosine A_{2A} receptor agonist into the nucleus

accumbens leads to a decreased locomotion (Barraco et al., 1993; Fredholm et al., 1999; Hauber and Munkle, 1997). Therefore, caffeine perhaps combines to A₁ receptor more preferentially than A_{2A} receptor with high concentration in the above situation, or there is some competition between A₁ and A_{2A} receptors to connect to caffeine.

Wanke et al (2008) reported that caffeine increased the yeast lifespan by releasing Rim15 from target of rapamycin complex 1(TORC1)-Sch9-mediated inhibition and finally inhibited TORC1. The Rim15-TORC1-Sch9 kinase cascade is evolutionarily conserved, which means caffeine may extend longevity in insects. Sutphin et al (2012) proposed that caffeine increased yeast lifespan through activating DAF-16 and reducing insulin/igf-1-like signaling, and then improved the diet-induced insulin resistance. The caffeine screen also showed that caffeine increased the life span of yeast (Lublin et al., 2011).

	ZT0	ZT2	ZT4	ZT6	ZT8	ZT10	ZT12	ZT14	ZT16	ZT18	ZT20	ZT22
0'00''-4'59''												
5'00''-9'59''												
10'00''-14'59''												
15'00''-19'59''												
20'00''-24'59''												
25'00''-29'59''												
30'00''-34'59''												
35'00''-39'59''												
40'00''-44'59''												
45'00''-49'59''												
50'00''-54'59''												
55'00''-59'59''												

Fig. 3 An example of analysis sheet for rest (black) and activity (white) based on video recording from behavior of one nymph drinking 200 μ g/ml melatonin under LD 12:12. ZT0-ZT10, the light-off period; ZT12-ZT22, the light-on period. Video was analyzed every 5 min and 12 phases was analyzed totally for each hour.

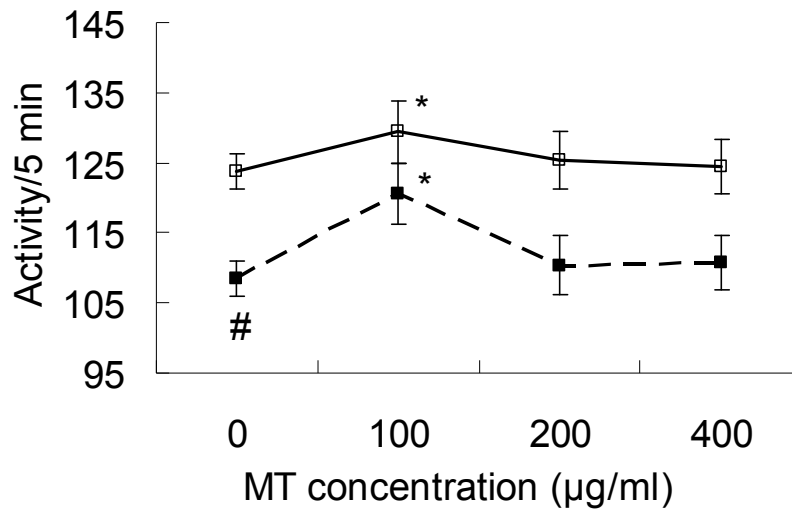


Fig. 4 Activity of nymphs reared under LD 16:8 (open squares) and LD 12:12 (solid squares and broken line) after drinking melatonin and water. Each point represents the mean $\pm$ SD. * $p < 0.05$, compared to the respective control (Fisher's LSD *post hoc* test following by a one-way ANOVA). # $p < 0.05$, significantly different between two photoperiods (two-way ANOVA, photoperiod $\times$ dose). MT, melatonin.

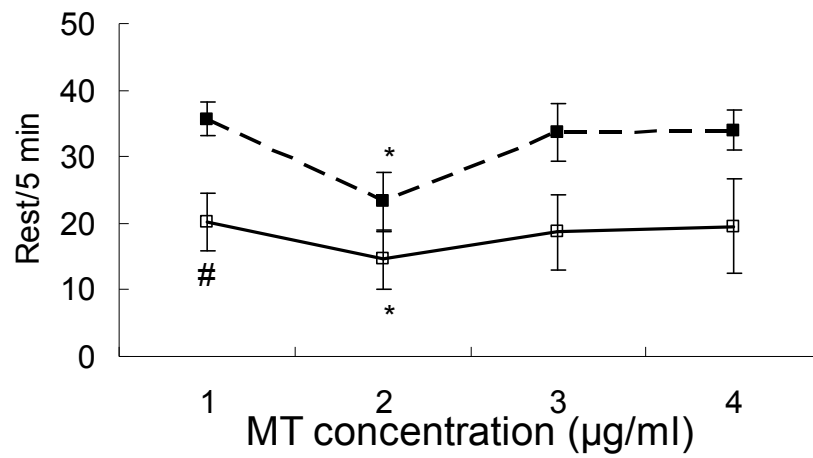


Fig. 5 Rest incidence of nymphs reared under LD 16:8 (open squares) and LD 12:12 (solid squares and broken line) after drinking melatonin and water. Each point represents the mean $\pm$ SD. * $p < 0.05$, compared to the respective control (Fisher's LSD *post hoc* test following by a one-way ANOVA). # $p < 0.05$, significantly different between two photoperiods (two-way ANOVA, photoperiod $\times$ dose). MT, melatonin.

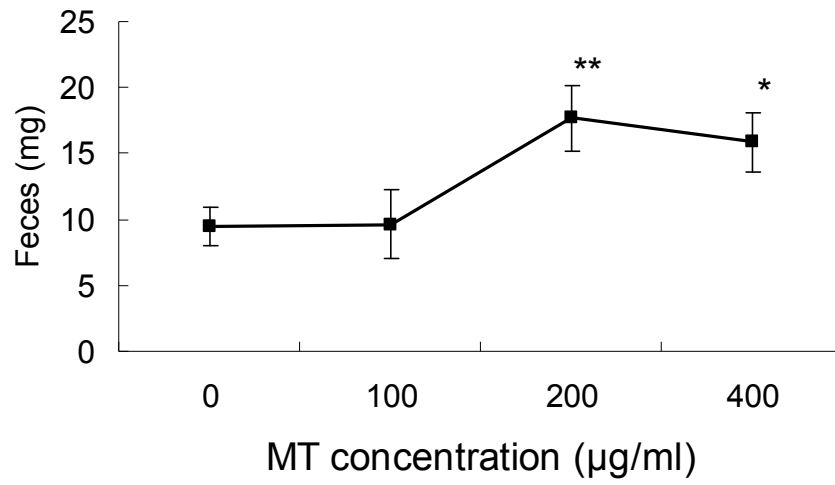


Fig. 6 Feces production of nymphs reared under LD 12:12 after drinking melatonin and water. Each point represents the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, compared to the respective control (Fisher's LSD *post hoc* test following by a one-way ANOVA). MT, melatonin.

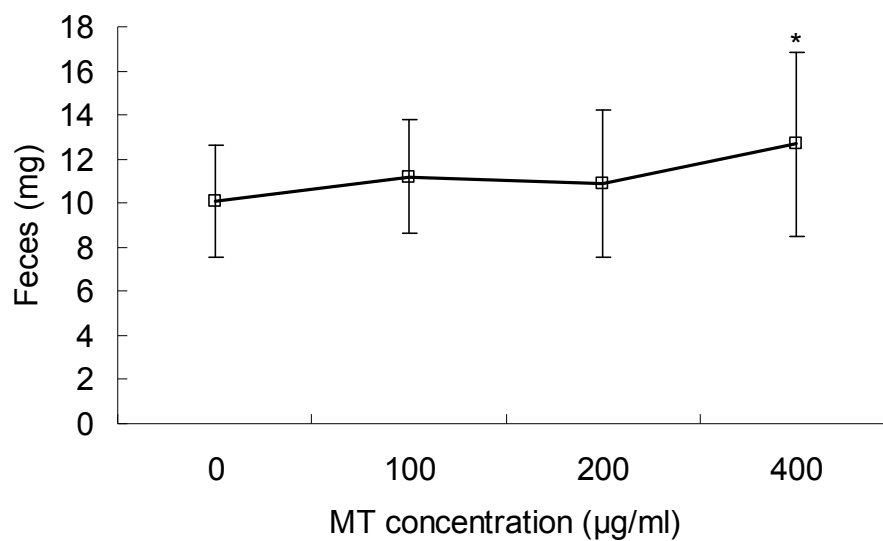


Fig. 7 Rest of nymphs under LD 16:8, after drinking melatonin and water.

Each point represents the mean $\pm$ SD. * $p < 0.05$, compared to the respective control (Fisher's LSD *post hoc* test following by a one-way ANOVA). MT, melatonin.

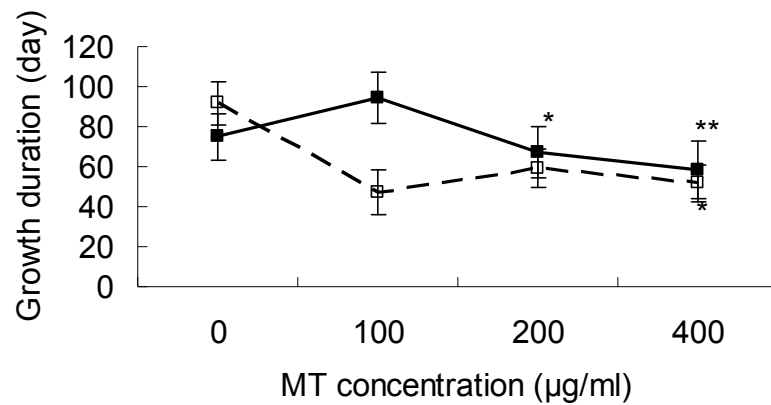


Fig. 8 Growth duration of nymph stage under LD 16:8 (open squares) and LD 12:12 (solid squares and broken line) after drinking melatonin and water. Each point represents the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, compared to the respective control (Fisher's LSD *post hoc* test following by a one-way ANOVA). MT, melatonin.

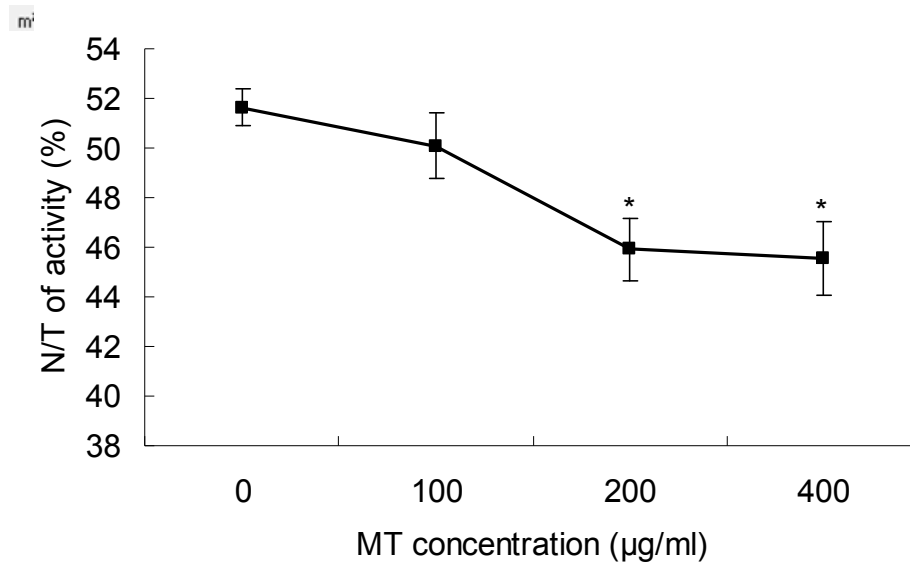


Fig. 9 Fraction of night activity of nymphs under LD 12:12 after drinking melatonin and water. Each point represents the mean $\pm$ SD. * $p < 0.05$, compared to the respective control (Fisher's LSD *post hoc* test following by a one-way ANOVA). MT, melatonin.

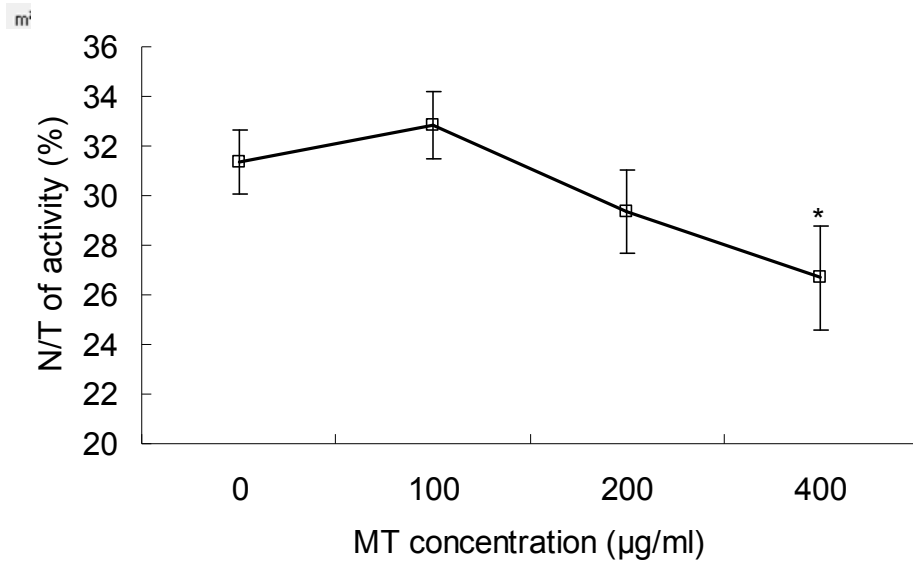


Fig. 10 Fraction of night activity of nymphs under LD 16:8 after drinking melatonin and water. Each point represents the mean $\pm$ SD. * $p < 0.05$, compared to the respective control (Fisher's LSD *post hoc* test following by a one-way ANOVA). MT, melatonin.

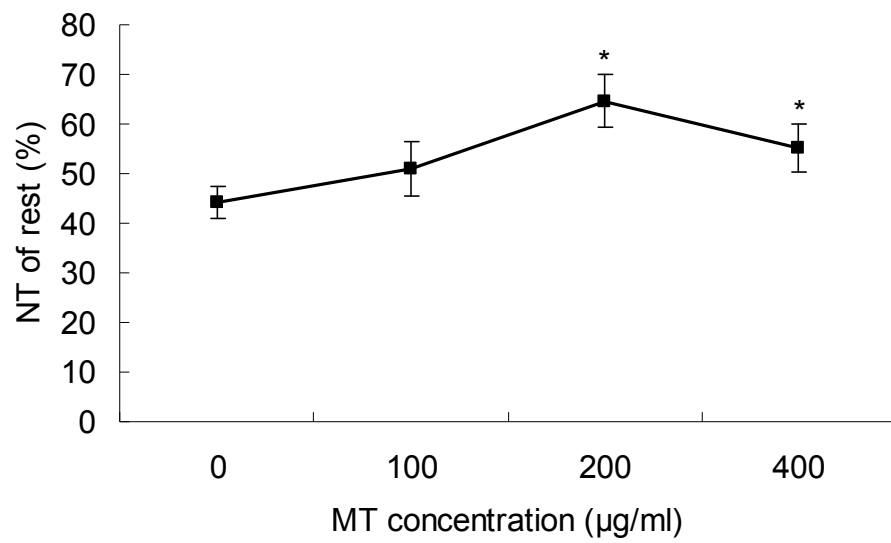


Fig. 11 Fraction of night rest of nymphs under LD 12:12 after drinking melatonin and water. Each point represents the mean $\pm$ SD. * $p < 0.05$, compared to the respective control (Fisher's LSD *post hoc* test following by a one-way ANOVA). MT, melatonin.

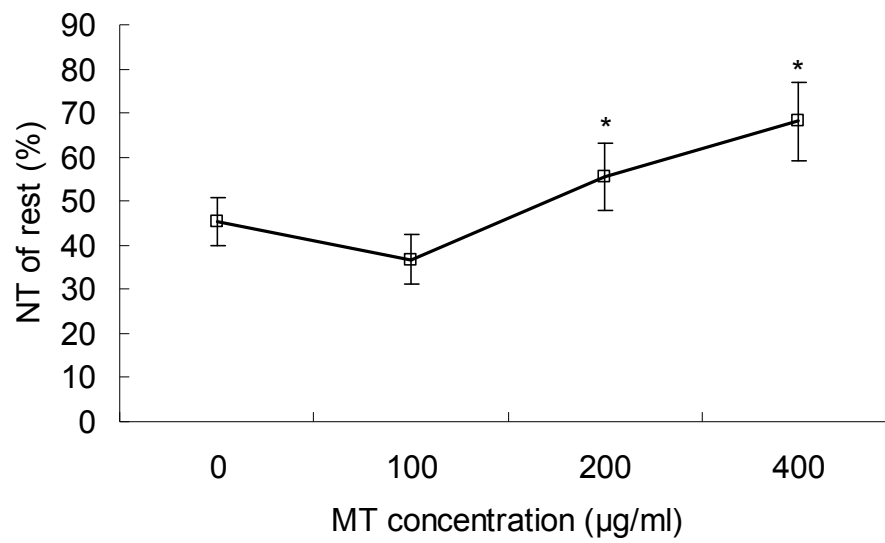


Fig. 12 Fraction of night rest of nymphs under LD 16:8 after drinking melatonin and water. Each point represents the mean $\pm$ SD. * $p < 0.05$, compared to the respective control (Fisher's LSD *post hoc* test following by a one-way ANOVA). MT, melatonin.

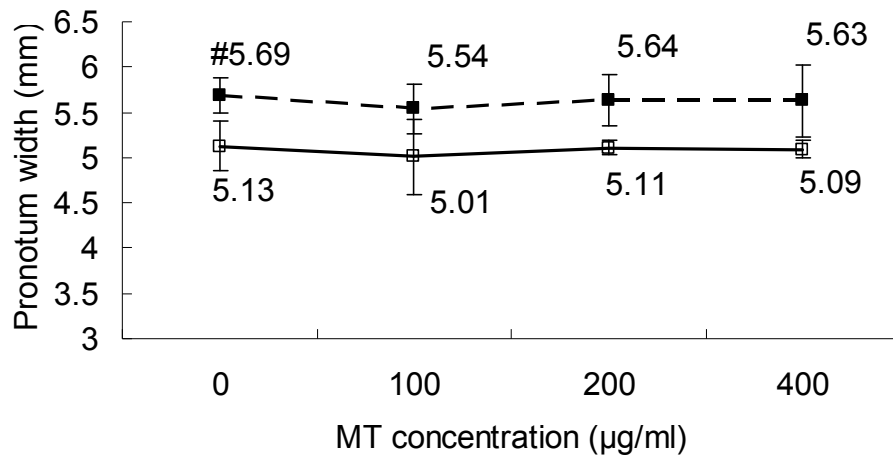


Fig. 13 Pronotum width of males that emerged from nymphs under LD 16:8 (open squares) and LD 12:12 (solid squares and broken line) after drinking melatonin and water, respectively. Each point represents the mean $\pm$ SD. The values under different treatments were inserted near each point. # $p < 0.05$, significantly different between two photoperiods (two-way ANOVA, photoperiod $\times$ dose). MT, melatonin.

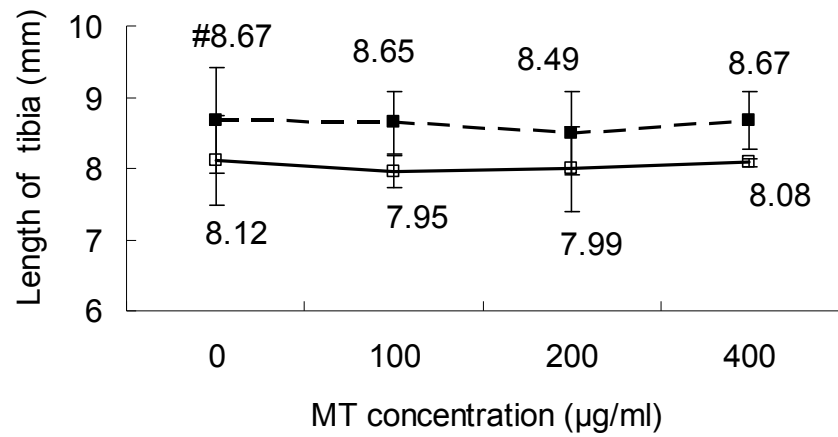


Fig. 14 Metathoracic tibia length of males that emerged from nymphs under LD 16:8 (open squares) and LD 12:12 (solid squares and broken line) after drinking melatonin and water, respectively. Each point represents the mean $\pm$ SD. The values under different treatments were inserted near each point. # $p < 0.05$, significantly different between two photoperiods (two-way ANOVA, photoperiod $\times$ dose). MT, melatonin.

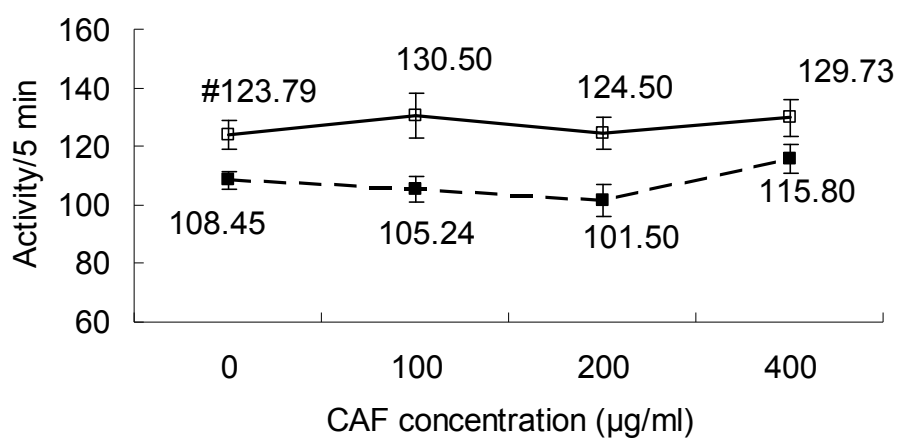


Fig. 15 Activity of nymphs reared under LD 16:8 (open squares) and LD 12:12 (solid squares and broken line) after drinking caffeine and water. Each point represents the mean $\pm$ SD. The values under different treatments were inserted near the each point. [#] $p < 0.05$, significantly different between two photoperiods (two-way ANOVA, photoperiod $\times$ dose). CAF, caffeine.

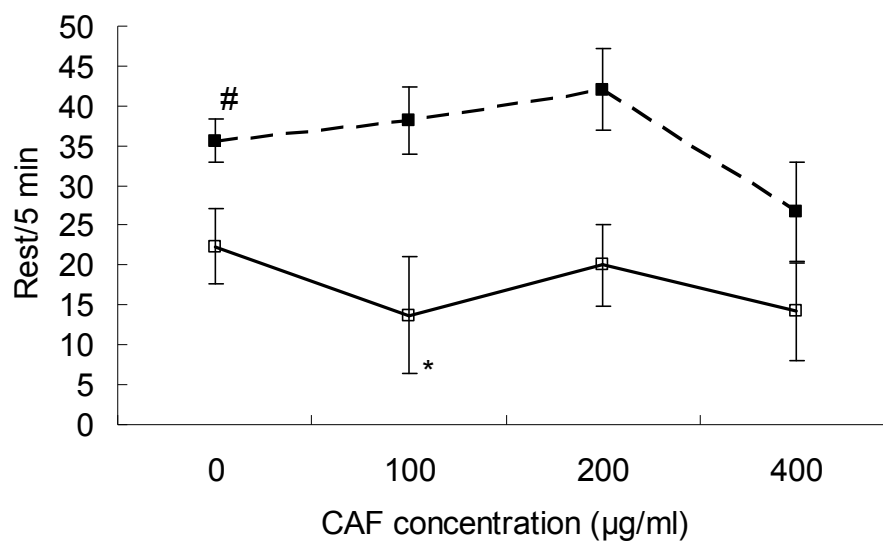


Fig. 16 Rest of nymphs under LD 16:8 (open squares) and LD 12:12 (solid squares and broken line) after drinking caffeine and water. Each point represents the mean $\pm$ SD. * $p < 0.05$, compared to the respective control (Fisher's LSD *post hoc* test following by a one-way ANOVA). [#] $p < 0.05$, significantly different between two photoperiods (two-way ANOVA, photoperiod $\times$ dose). CAF, caffeine.

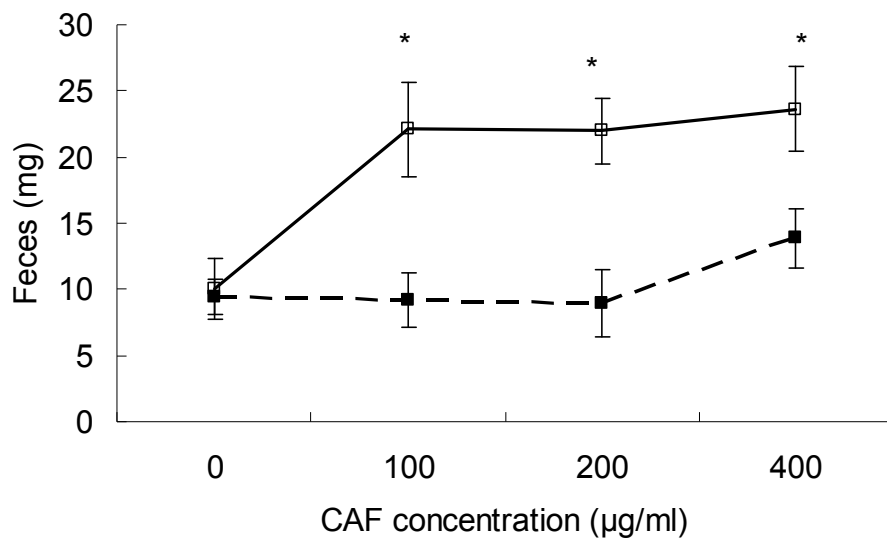


Fig. 17 Feces production of nymphs under LD 16:8 (open squares) and LD 12:12 (solid squares and broken line) after drinking caffeine and water. Each point represents the mean $\pm$ SD. * $p < 0.05$, compared to the respective control (Fisher's LSD *post hoc* test following by a one-way ANOVA). CAF, caffeine.

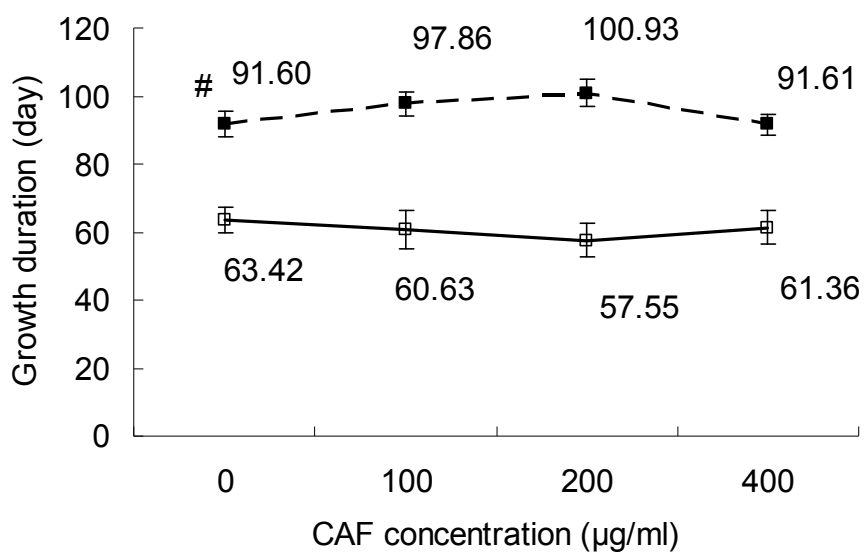


Fig. 18 Growth duration of nymphs reared under LD 16:8 (open squares) and LD 12:12 (solid squares and broken line) after drinking caffeine and water. Each point represents the mean $\pm$ SD. The values under different treatments were inserted near the each point. [#] $p < 0.05$, significantly different between two photoperiods (two-way ANOVA, photoperiod $\times$ dose). CAF, caffeine.

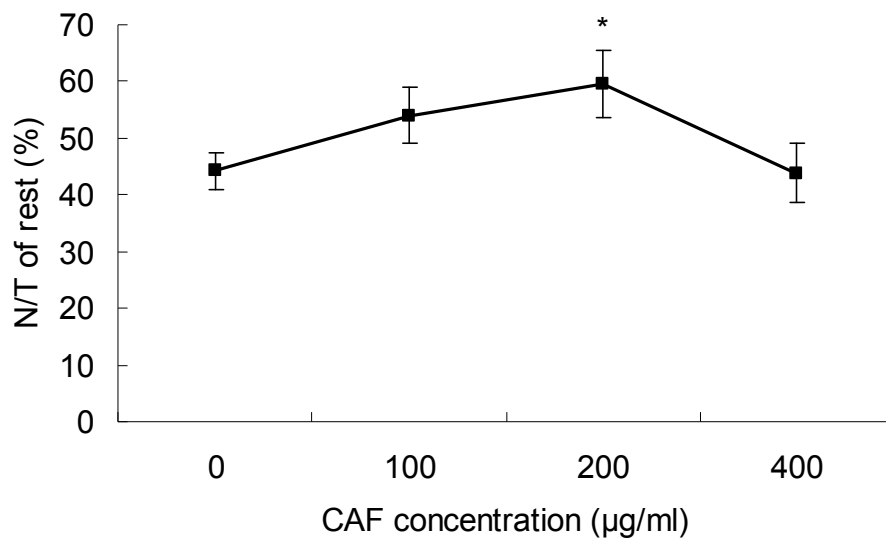


Fig. 19 Fraction of night rest of nymphs reared under LD 12:12 after drinking caffeine and water. Each point represents the mean $\pm$ SD. * $p < 0.05$, compared to the respective control (Fisher's LSD *post hoc* test following by a one-way ANOVA). CAF, caffeine.

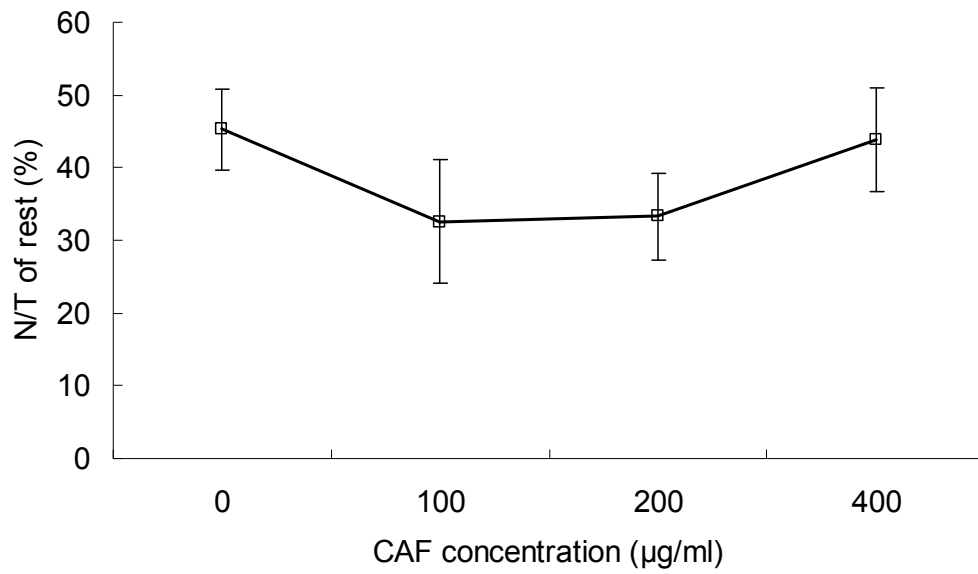


Fig. 20 Fraction of night rest of nymphs reared under LD 16:8 after drinking caffeine and water. Each point represents the mean $\pm$ SD. CAF, caffeine.

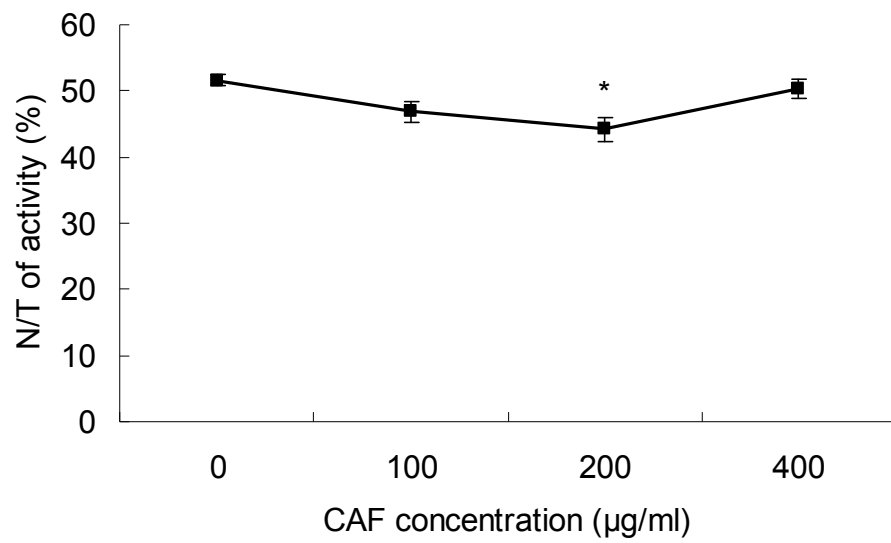


Fig. 21 Fraction of night activity of nymphs reared under LD 12:12 after drinking caffeine and water. Each point represents the mean $\pm$ SD. * $p < 0.05$, compared to the respective control (Fisher's LSD *post hoc* test following by a one-way ANOVA). CAF, caffeine.

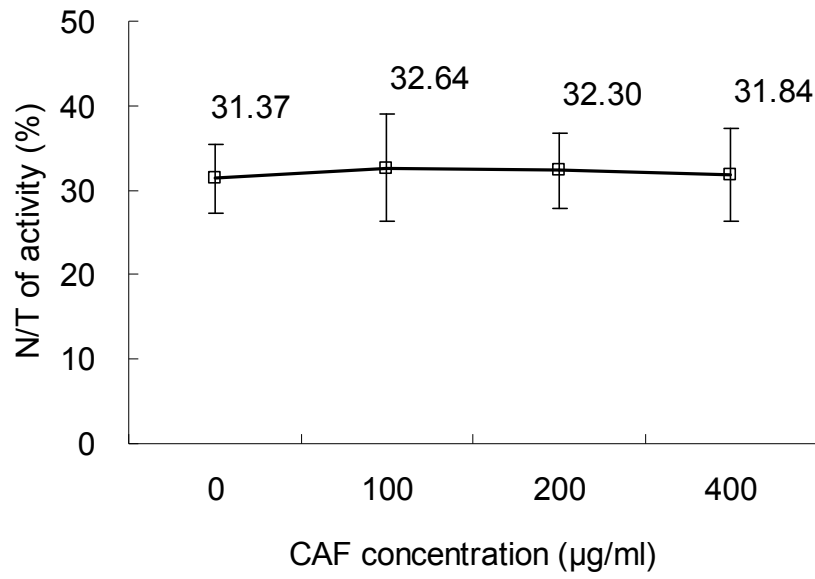


Fig. 22 Fraction of night activity of nymphs reared under LD 16:8 after drinking caffeine and water. Each point represents the mean $\pm$ SD. The values under different treatments were inserted near each point. CAF, caffeine.

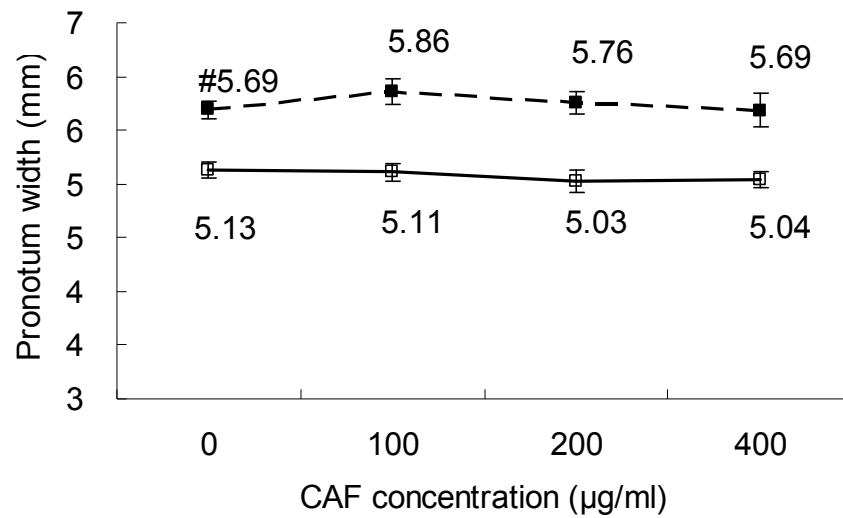


Fig. 23 Pronotum width of males that emerged from nymphs reared under LD 16:8 (open squares) and LD 12:12 (solid squares and broken line) after drinking caffeine and water, respectively. Each point represents the mean $\pm$ SD. The values under different treatments were inserted near each point. [#] $p < 0.05$, significantly different between two photoperiods (two-way ANOVA, photoperiod $\times$ dose). CAF, caffeine.

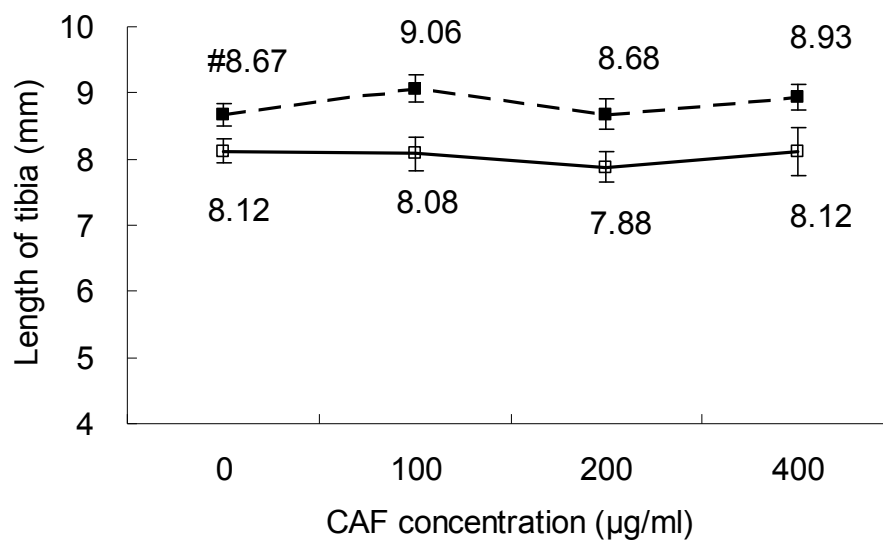


Fig. 24 Metathoracic tibia length of males that emerged from nymphs reared under LD 16:8 (open squares) and LD 12:12 (solid squares and broken line) after drinking caffeine and water, respectively. Each point represents the mean $\pm$ SD. The values under different treatments were inserted near each point. # $p < 0.05$, significantly different between two photoperiods (two-way ANOVA, photoperiod $\times$ dose). CAF, caffeine.

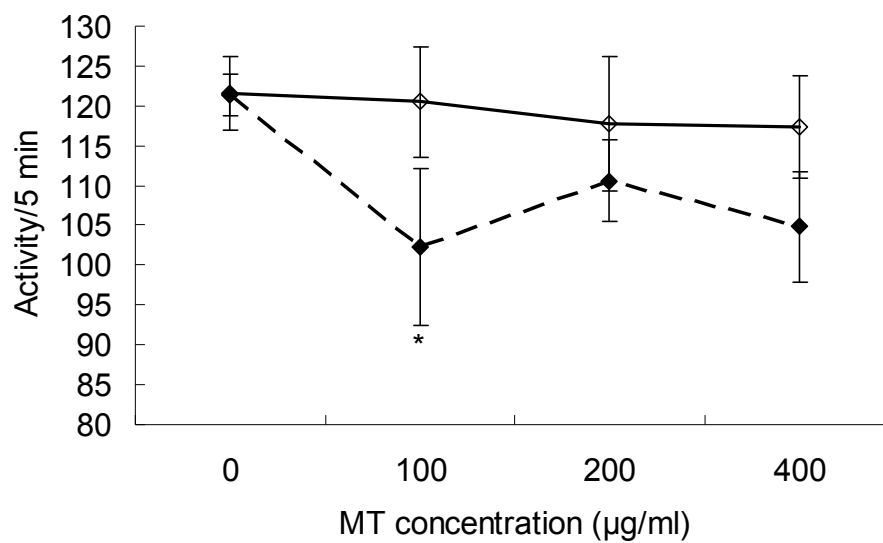


Fig. 25 Activity of males (solid diamond and broken line) and females (open diamond) after drinking melatonin and water, respectively. Each point represents the mean $\pm$ SD. * $p < 0.05$, compared to the respective control (Fisher's LSD *post hoc* test following by a one-way ANOVA). MT, melatonin.

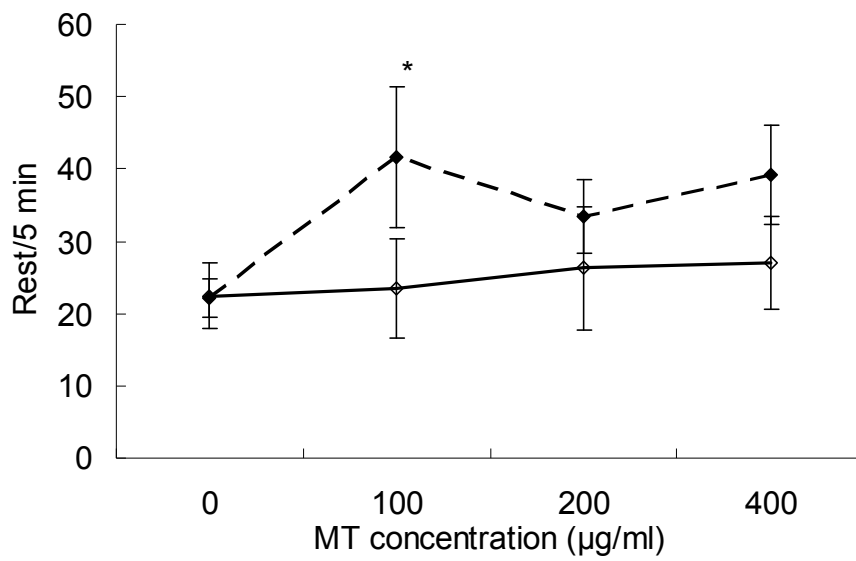


Fig. 26 Rest of males (solid diamond and broken line) and females (open diamond) after drinking melatonin and water, respectively. Each point represents the mean $\pm$ SD. * $p < 0.05$, compared to the respective control (Fisher's LSD *post hoc* test following by a one-way ANOVA). MT, melatonin.

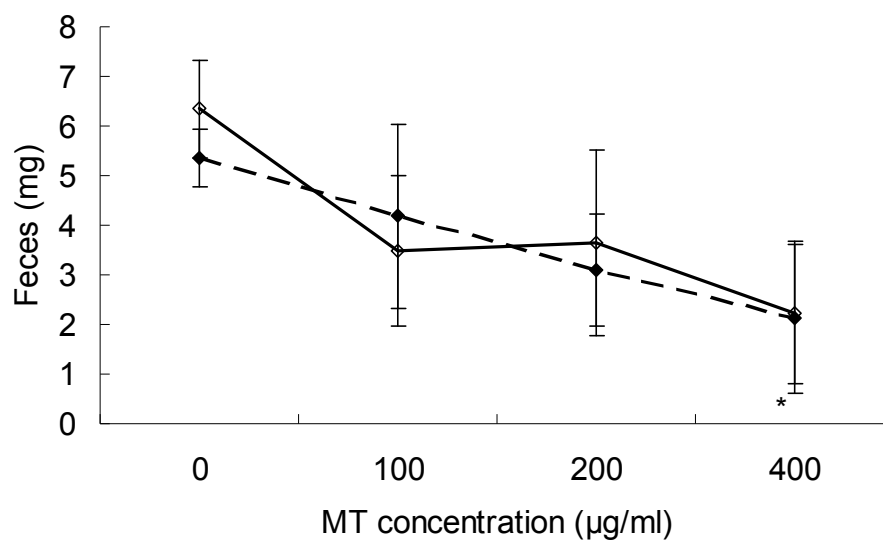


Fig. 27 Feces produced by males (solid diamond and broken line) and females (open diamond) after drinking melatonin and water, respectively. Each point represents the mean $\pm$ SD. * $p < 0.05$, compared to the respective control (Fisher's LSD *post hoc* test following by a one-way ANOVA). MT, melatonin.

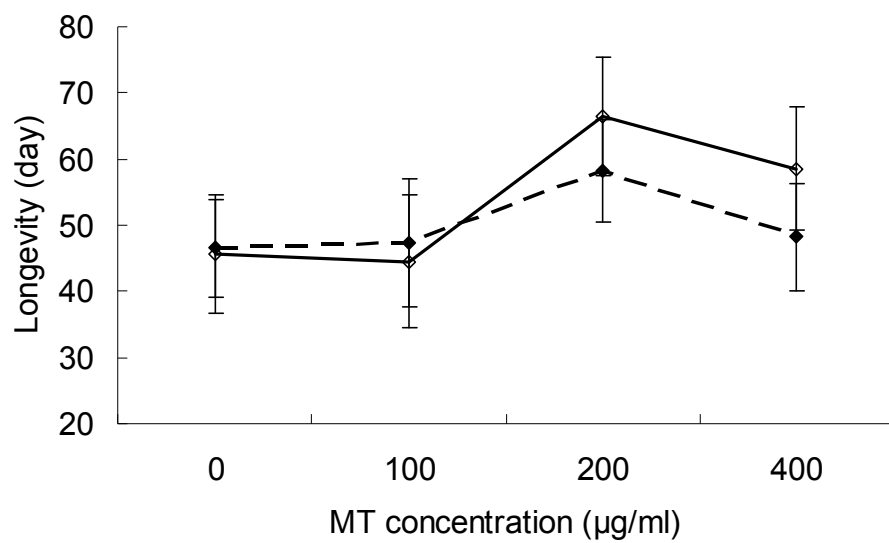


Fig. 28 The duration of adult stage in males (solid diamond and broken line) and females (open diamond) after drinking melatonin and water, respectively. Each point represents the mean $\pm$ SD. MT, melatonin.

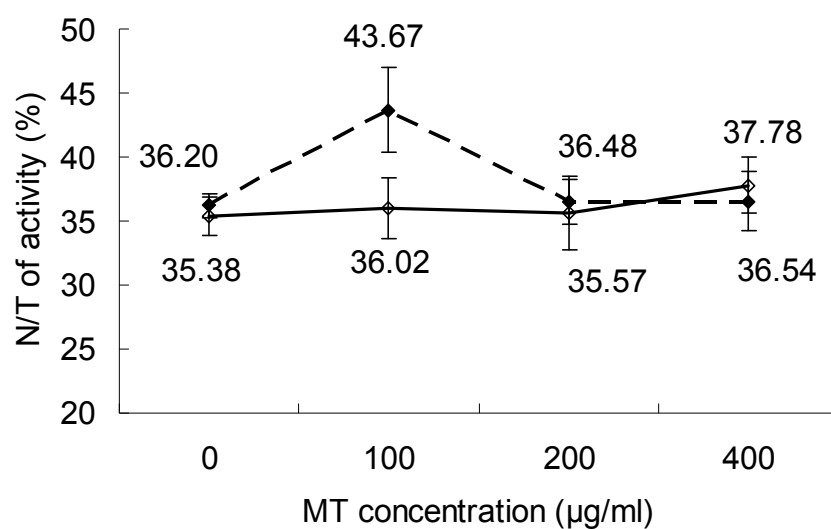


Fig. 29 Fraction of night activity of males (solid diamond and broken line) and females (open diamond) after drinking melatonin and water, respectively. Each point represents the mean $\pm$ SD. The values under different treatments were inserted near each point. MT, melatonin.

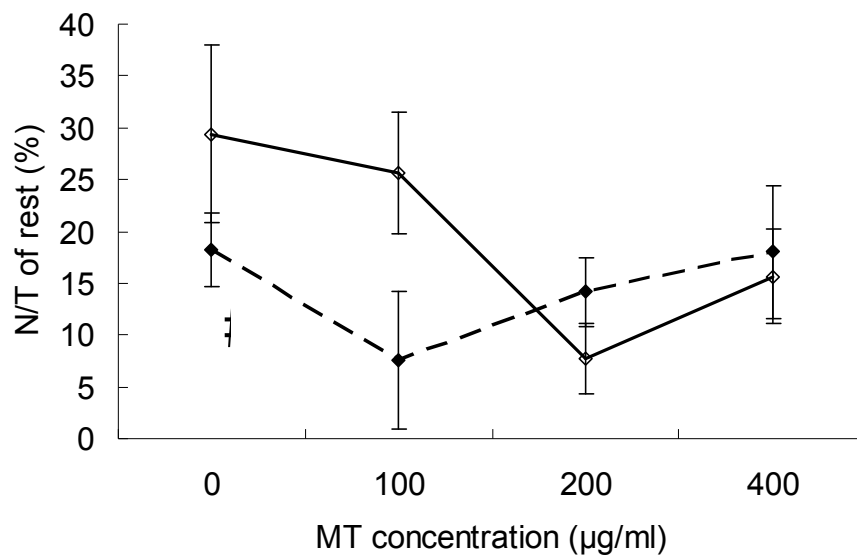


Fig. 30 Fraction of night rest of males (solid diamond and broken line) and females (open diamond) after drinking melatonin and water, respectively. Each point represents the mean $\pm$ SD. The values under different treatments were inserted near each point. [#] $p < 0.05$, significantly different between two photoperiods (two-way ANOVA, photoperiod $\times$ dose). CAF, caffeine.

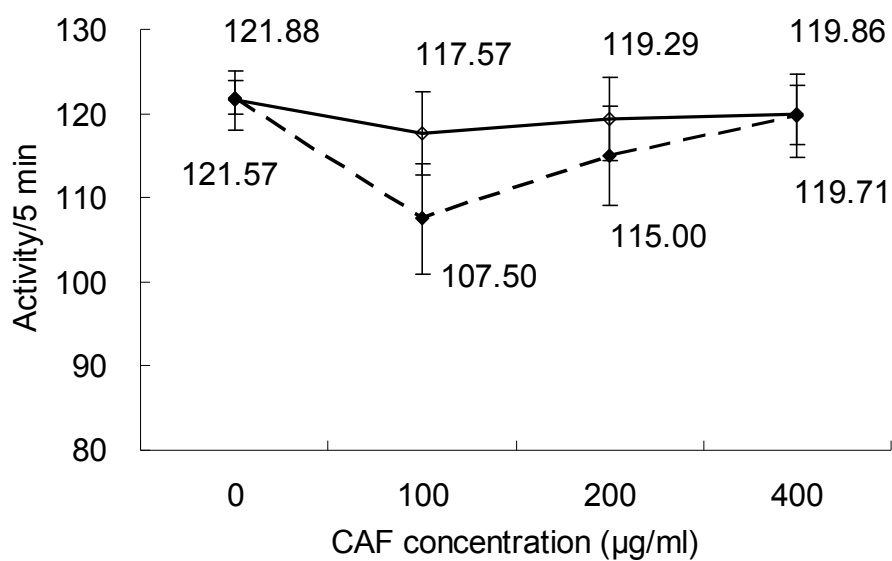


Fig. 31 Activity of males (solid diamond and broken line) and females (open diamond) after drinking caffeine and water, respectively. Each point represents the mean $\pm$ SD. The values under different treatments were inserted near the each point. CAF, caffeine.

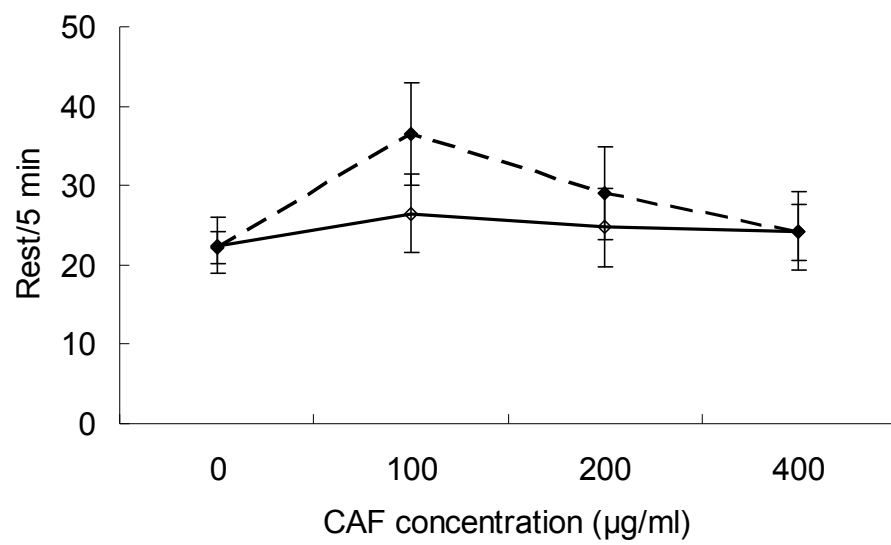


Fig. 32 Rest of males (solid diamond and broken line) and females (open diamond) after drinking caffeine and water, respectively. CAF, caffeine.

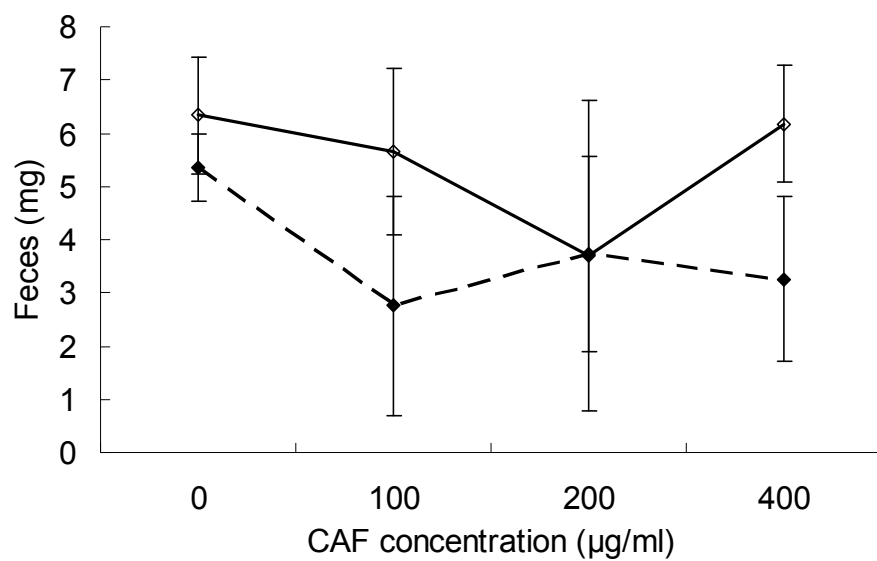


Fig. 33 Feces of males (solid diamond and broken line) and females (open diamond) after drinking caffeine and water, respectively. CAF, caffeine.

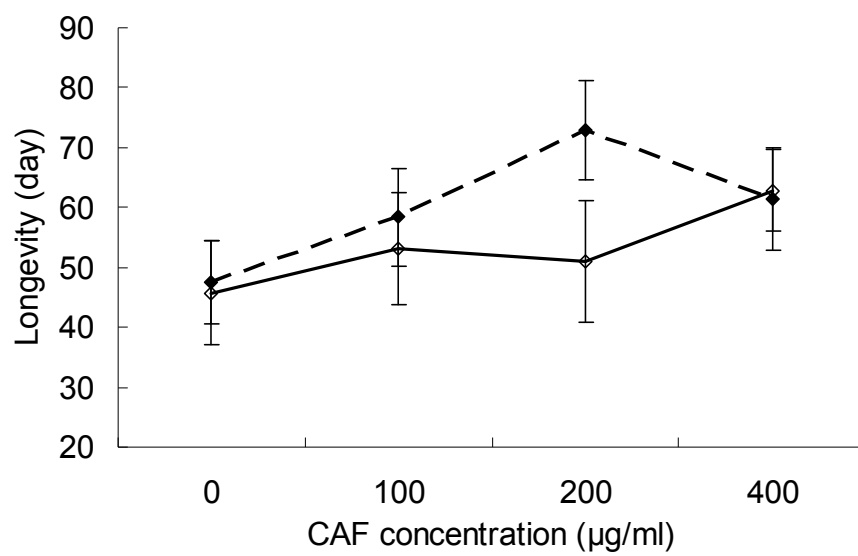


Fig. 34 Growth duration of adult stage of males (solid diamond and broken line) and females (open diamond) after drinking caffeine and water, respectively. CAF, caffeine.

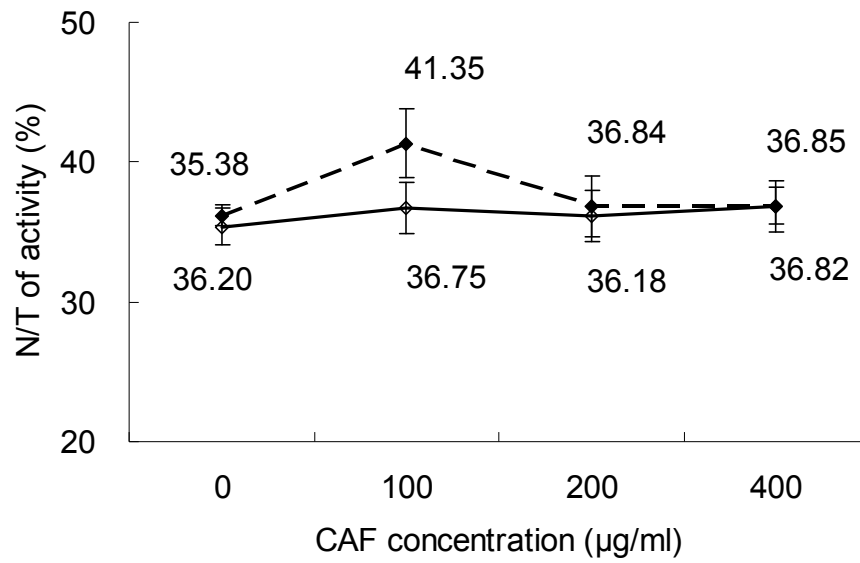


Fig. 35 Fraction of night activity of males (solid diamond and broken line) and females (open diamond) after drinking caffeine and water, respectively. Each point represents the mean $\pm$ SD. The values under different treatments were inserted near each point. CAF, caffeine.

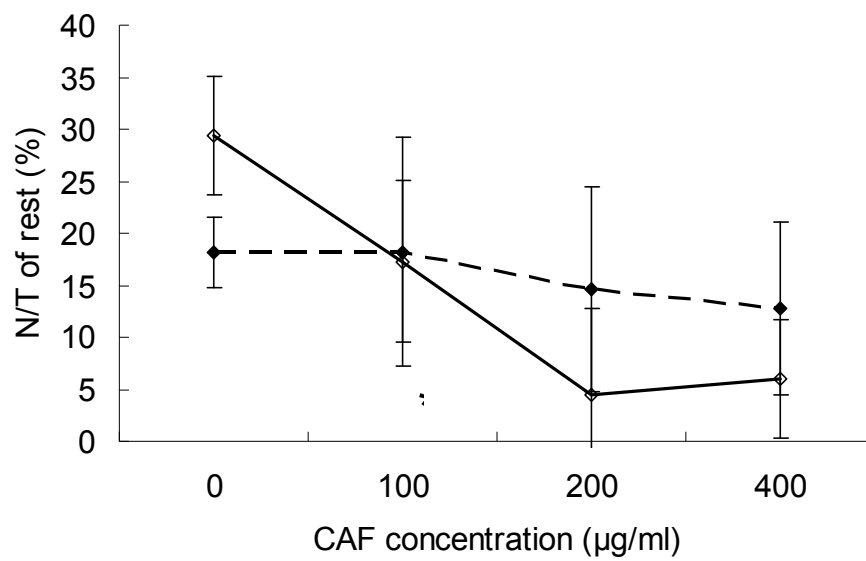


Fig. 36 Fraction of night rest of males (solid diamond and broken line) and females (open diamond) after drinking caffeine and water, respectively. Each point represents the mean $\pm$ SD. * $p < 0.05$, compared to the respective control (Fisher's LSD *post hoc* test following by a one-way ANOVA). CAF, caffeine.

Chapter 3

Effects of melatonin and caffeine on locomotor activity and circadian rhythm in the cricket

3.1 Abstract

To clarify the effects of melatonin and caffeine on circadian rhythm in the crickets, we studied the locomotor activity of the cricket, *Gryllus vavivus*. Both melatonin and caffeine affected the parameters of circadian rhythm. In details, melatonin decreased activity counts, advanced acrophase, increased amplitude and the fraction of night activity in both males and females in LD12:12. In DD, melatonin again decreased activity counts and increased amplitude, but the acrophase lagged behind, and the free running period increased, the fraction of night activity decreased. The same increased amplitude and decreased activity indicate that melatonin suppresses activity and intensifies the circadian rhythm. Meanwhile, caffeine affected in the similar way, which decreased the activity counts, advanced acrophase, increased amplitude and the fraction of night activity in both sexes in LD 12:12. But in DD, caffeine decreased the amplitude in both sexes, though it also decreased the activity counts and the fraction of night activity. Besides, the acrophase advanced in males while moving behind in females. The difference result of amplitude indicates that caffeine may functions depending on light conditions and synchronizes the light-dark circles. The decreased fraction of night activity and the increased arrhythmic number

after transferring to the dark indicate that caffeine disorders the circadian rhythm in the constant dark. The effects of two medicines have no significant difference between two sexes.

Key words: melatonin, caffeine, circadian rhythm, locomotor activity, light-dark cycle, the constant dark

3.2 Introduction

The effect on circadian rhythms of physiological phenomena is one of the overwhelming studies on melatonin. Oral administration or injection of melatonin to mammals, birds and lizards has been shown to affect locomotor activity rhythms (Redman et al., 1983; Underwood, 1986; Heigl and Gwinner 1992, 1994; Singaravel et al., 1998; Sharma et al., 1999). Melatonin is involved in the phototransduction pathway to the circadian pacemaker (Yamano et al., 2000), and has been detected locating in the ocellar nerve in the insect, *Periplaneta Americana* (Takeda et al., 1988) which suppresses nocturnal activity in insects. Melatonin may be a coupling modulator of constituent oscillators (Yamano et al., 2000). Besides, the study shows that melatonin modulates the circadian phase by activating MT₁ and MT₂ melatonin receptors (Margarita, 2007).

Caffeine lengthens circadian rhythms in mice in the constant dark and delayed the phase of the mouse liver clock (Oike et al., 2011). It affected the clock genes expression and is tissue dependent (Sherman et al., 2011). In addition caffeine depressed melatonin in humans at night (Wright et al., 1997). Its function on circadian

rhythm has not been reported in insects, though it has been reported that caffeine disordered the sleep and motion (American Psychiatric Association, 1994).

In this chapter, we study the effects of melatonin and caffeine on circadian rhythm of locomotor activity in males and females of the cricket, *Gryllus vavivus*. The activity level, circadian parameters were analyzed in both males and females given three different concentrations of two medicines and a control.

3.3 Material and methods

3.3.1 Animals

Adult male and female crickets, *Gryllus vavivus* were used for the experiment. They were reared under LD 16:8 at 20°C and fed with mouse food (MF, ORIENTAL YEAST Co. Ltd., Tokyo, Japan) and water.

3.3.2 Recording of locomotor activity

Locomotor activity of individual animals was recorded with an activity chamber made of transparent plastic box (194×105×28mm) with the plastic shelter. Activities were accumulated every 6 min to be the number of times an infra-red beam (940-950nm) across the box was broken, and then fed into a computer that summed a total number of animal's movement. The methodology is similar to that described in Yamano et al. (2000). Melatonin was dissolved firstly in ethanol and then in distilled water to desired concentrations. Caffeine was dissolved in distilled water to the desired concentrations directly and supplied to the adult crickets. Water was used as control. The activity chambers were placed in an incubator in which temperature was

kept at 20°C. Adults were entrained to LD 12:12 for 10 days and then transferred to DD. The forewings of males were clipped to prevent interference between insects by stridulation.

Two groups of experiments were prepared for each medicine test. In the first group, only males were tested with control, 100 µg/ml, 200 µg/ml and 400 µg/ml concentrations of medicines and water. In the second group, only females were tested. In both groups the procedure was the same, virgin around 20 day old male or female crickets were put into 40 boxes (one individual per box) for each experiment.

3.3.3 Data analysis

Actograms were double-plotted as conventionally practiced using a computer (Fig. 1A; Koga et al., 2005). Amplitudes and acrophases were estimated from the cosine function, $f(t) = M + A \cos(\omega t + \phi)$, which defines three characteristic parameters of a circadian rhythm: the mesor (M), amplitude (A), and acrophase (ϕ). The amplitude was the distance from the rhythmic mean to the peak or trough of a mathematical model. Acrophase was the time period during which the highest values were expected (Fig. 1B, Tanakadate et al., 1991). The cycle length of the entrained rhythm (τ_{LD}) and free-running period (τ_{DD}) were analyzed by chi-square periodogram analysis (Fig. 1C; Tanakadate et al., 1991). The fraction of night-time activity (N/T) in both LD and DD were calculated. In LD, the length of night was 12 h from ZT 0 to ZT 12, while the length of subjective night was calculated from the free running period starting from the circadian time (CT 12) in DD. These values were shown as means $\pm$ standard deviation (SD). To determine the effect of medicines on circadian parameters

(acrophase, amplitude, activity level, τ_{LD} , τ_{DD} , N/T_{LD} , and N/T_{DD}), a one-way ANOVA with Fisher's LSD for multiple comparisons and two-way ANOVA were subsequently

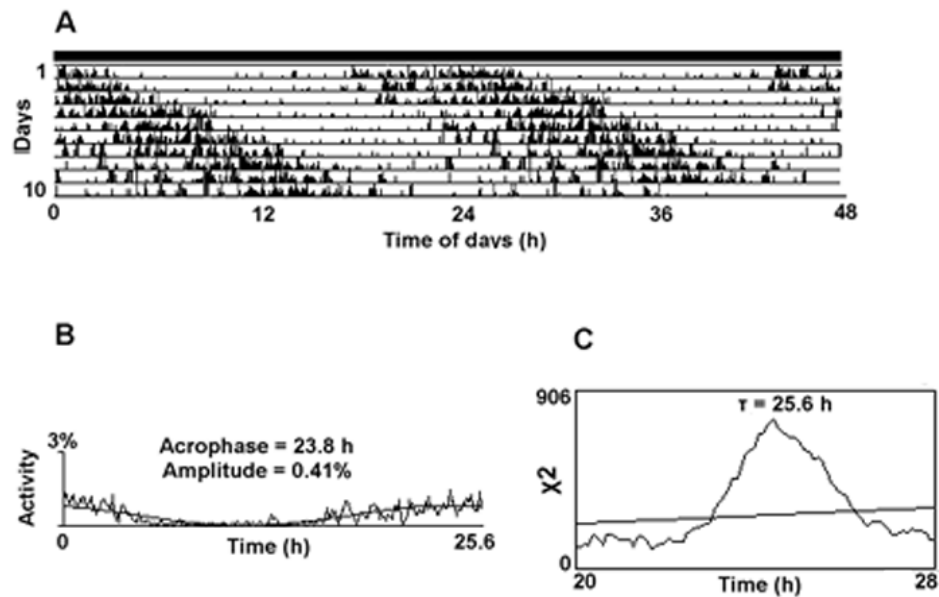


Fig. 1. A, an example of a double-plotted actogram of one female in DD for 10 days at 20°C. B, acrophase and amplitude. C, chi square periodogram analysis, the ascending line represents a confidence level of 99% and the major peak with its maximum at the free running period ($\tau = 25.6$ h).

performed to determine differences in the effects of melatonin or caffeine between the two sexes (sex $\times$ dose) on the circadian rhythm. These values were based on cumulative data for ten cycles/individual before and after the LD-DD transition.

3.4 Results

3.4.1 *Effects of melatonin on locomotor rhythm when entrained to LD 12:12*

a) male

Locomotor activity of adult male crickets under LD 12:12 was double plotted (Fig. 2, A1-A4). The animals were reared in LD 12:12 for the first 10 days then transferred to DD for another 10 days. The activity level, acrophase, amplitude, τ_{LD} and fraction of night-time activity (N/T_{LD}) were calculated for each concentration of melatonin (table 1). In males, the activity level decreased largely with melatonin treatments compared with control, the effect was highly significant while there was no significant difference between caffeine drinking groups (LSD, $p < 0.01$, Fig. 3).

After drinking melatonin, the acrophase was slightly phase advanced compared to the control group and 100 $\mu\text{g/ml}$ treatment had the highest effect. But it was interestingly delayed in 200 $\mu\text{g/ml}$ group (Table 1). The amplitude increased in melatonin drinking groups, especially that drinking 400 $\mu\text{g/ml}$ melatonin (Table 1). N/T_{LD} increased as well, and significantly in 400 $\mu\text{g/ml}$ concentration group (LSD, $p < 0.05$, Table 1).

b) female

The locomotor activity of adult female crickets was also double plotted (Fig. 2 B1-B4). Similar with males, the activity counts of the individuals drinking melatonin decreased significantly (Fig. 4), especially in 200 and 400 $\mu\text{g/ml}$ groups (LSD, $p < 0.05$, Fig. 4, Table 1), and 200 $\mu\text{g/ml}$ group decreased activity most significantly. The acrophase of females drinking melatonin was also earlier than the control group and 200 $\mu\text{g/ml}$ treatment had the significant effect (LSD, $p < 0.05$). The amplitude of females had the same increased trend and 400 $\mu\text{g/ml}$ melatonin increased most (Table 1). For females, N/T_{LD} increased as well, though not significantly (Table 1).

The effects of melatonin on males and females had the same trend when they were entrained to LD 12:12. It decreased the activity level ($p < 0.01$, one-way ANOVA), shifted the acrophase earlier, increased the amplitude which responded that the activity centered and the phase moved to the night because N/T_{LD} increased. Drinking melatonin clearly affected these attributes. In the LD cycles, the acrophase of females from control was in the early day time, which was totally different from males. But after drinking melatonin, the acrophase appeared in the dark in females with 100 and 200 $\mu\text{g/ml}$ treatments (Table 1). Two-way ANOVA showed no significant difference between males and females with melatonin treatments (data not shown).

3.4.2. Effects of melatonin on the free-running rhythm in DD

Effects of melatonin on the free running period of males also examined. The individuals were transferred from LD cycles and tested in the constant dark for

another 10 days. In DD, the activity level also decreased dramatically both in males and females with the caffeine treatments (male, $p < 0.01$; female, $p < 0.01$, one-way ANOVA). And the same with the LD results, group drinking 200 $\mu\text{g/ml}$ caffeine had the highest activity counts among the three groups drinking caffeine (Fig. 4 and Fig. 5). The amplitude of both male and female in the constant dark increased, which showed that the activity became centered in the constant dark as well, though not significantly. On the other hand, the acrophase under the dark condition lagged behind in both males and females. τ_{DD} increased slightly both in males and females. The fraction of subjective night activity (N/T_{DD}) decreased in the constant dark (Table 2).

Like under LD 12:12, the effects of melatonin also had the same trend in both males and females under DD. But the acrophase moved behind, τ_{DD} increased and N/T_{DD} decreased in the constant dark, which were conversely to these under LD 12:12. Two-way ANOVA showed no significant difference between males and females with melatonin treatments (data not shown).

3.4.3 Effects of caffeine on locomotor rhythm when entrained to LD 12:12

Fig. 3 shows the examples of locomotor activity in both males and females with three concentrations of caffeine treatments and drinking water. The first 10 days were under LD 12:12. In males, locomotor activity levels were significantly lower in the caffeine-treated groups than in the control ($p < 0.01$, one-way ANOVA), but no significant difference was observed among the caffeine-treated groups, and activity level was slightly higher in the 200 $\mu\text{g/ml}$ caffeine group than the other caffeine-treated groups (Table 3, Fig. 6). Acrophase in the caffeine-drinking groups advanced, and amplitude increased especially in the 100 $\mu\text{g/ml}$ group (Table 3). τ_{LD}

became shorter in the caffeine treated groups, closing to 24h (Table 3). N/T_{LD} in the caffeine-treated groups was higher than in the control, and was especially significantly higher in the 100 $\mu\text{g/ml}$ group (LSD, $p < 0.05$, Table 3).

Activity counts were significantly lower in females drinking caffeine ($p < 0.01$, one-way ANOVA, Fig. 7), but no significant difference was observed among the caffeine-treated groups, however activity levels were significantly higher in the 200 $\mu\text{g/ml}$ group than two other caffeine-treated groups (Fig. 7). Acrophase in females drinking caffeine was also earlier than that of the control group, and significantly different in the 200 $\mu\text{g/ml}$ group (LSD, $p < 0.05$, Table 3). The amplitude of rhythms in females increased as well and significantly higher in the 200 $\mu\text{g/ml}$ group (LSD, $p < 0.05$, Table 3). τ_{LD} was significantly shorter and became closer to 24h in females drinking caffeine ($p < 0.05$, one-way ANOVA), and 200 $\mu\text{g/ml}$ group had the shortest τ_{LD} ($p < 0.05$, Fisher's LSD, Table 3). N/T_{LD} increased in all groups except for the 100 $\mu\text{g/ml}$ group, and significantly increased in the 200 $\mu\text{g/ml}$ group (LSD, $p < 0.05$, Table 3). τ_{LD} in males and females was closer to 24h in the caffeine treated groups, which indicated caffeine intensified the modulation of LD and synchronized the circadian rhythms under LD.

3.4.4 Effects of caffeine on the free-running rhythm in DD

The second 10 days of Fig. 3 shows the locomotor activity of both sexes in DD. Similar to that under LD 12:12, activity levels with caffeine treatments were significantly decreased ($p < 0.01$ in males; $p < 0.01$ in females, one-way ANOVA), and no significant differences were observed among the caffeine-treated groups and

activity levels were slightly higher in the 200 µg/ml group than the other caffeine-treated groups (Table 4, Fig. 6 and Fig. 7). In contrast, the amplitude of rhythm in both males and females was slightly decreased in DD (Table 4). According to the chi-square periodogram analysis, the number of insects with arrhythmic activity in DD increased in both males and females caffeine-treated groups (for males, $n = 1$ in 100 µg/ml, 3 in 200 µg/ml, 6 in 400 µg/ml; for females, $n = 8$ in 100 µg/ml, 5 in 200 µg/ml, 8 in 400 µg/ml), while no arrhythmic insects were reported in the control group. Decreased amplitude and increased arrhythmic number indicate that activity levels in DD were caffeine-disordered. On the other hand, acrophase in DD was different between males and females (Table 4). In males, acrophase was phase-advanced in the caffeine-treated groups while in females, it lagged behind as caffeine concentrations increased, except for the 100 µg/ml group (Table 4). In contrast to LD conditions, subjective N/T_{DD} in the free running period decreased in both males and females, with a significant decrease being observed in the 200 µg/ml group in males (LSD, $p < 0.05$, Table 4). Two-way ANOVA shows no significant difference of the effects between males and females (data not shown).

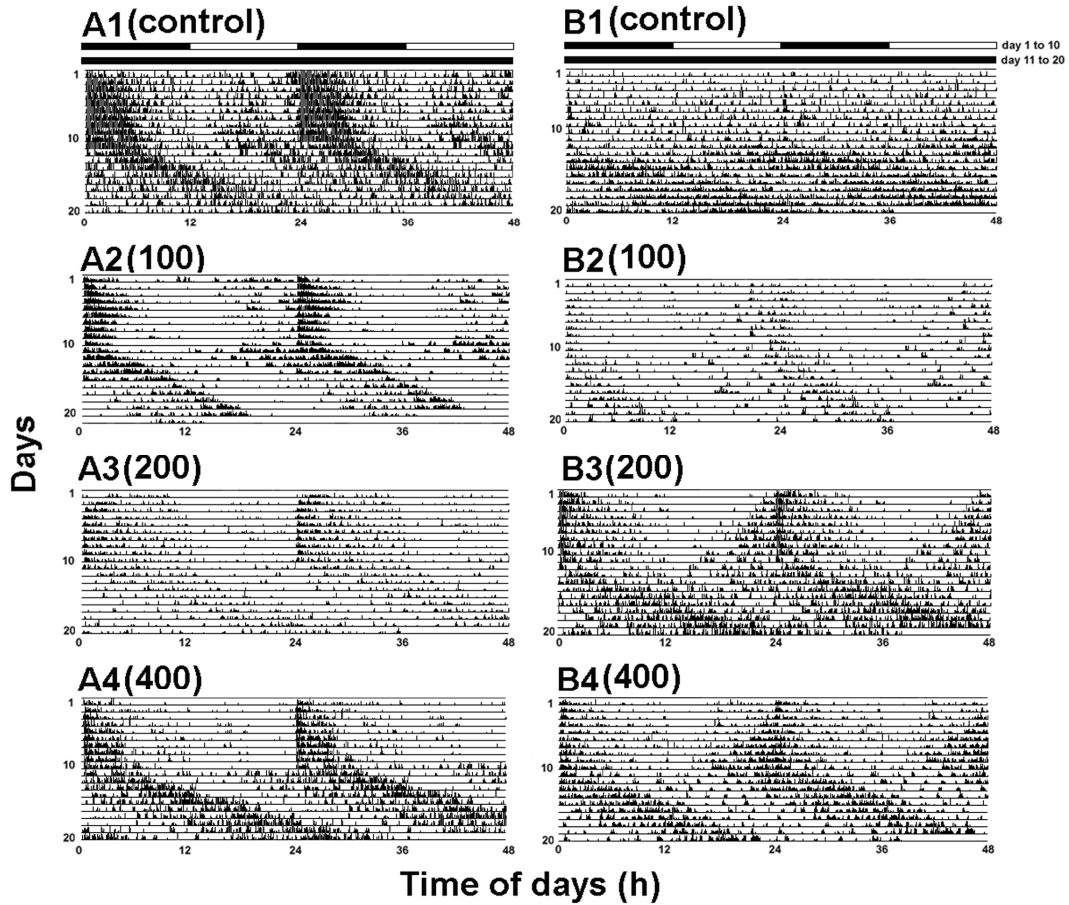


Fig. 2. Examples of locomotor activity patterns in males (A1–A4) and females (B1–B4) drinking melatonin and control. The Crickets were entrained under LD 12:12 for the first 10 days then transferred to DD for the second 10 days. A1, control; A2, with 100 $\mu\text{g}/\text{ml}$ melatonin; A3, with 200 $\mu\text{g}/\text{ml}$ melatonin; A4, with 400 $\mu\text{g}/\text{ml}$ melatonin; B1, control; B2, with 100 $\mu\text{g}/\text{ml}$ melatonin; B3, with 200 $\mu\text{g}/\text{ml}$ melatonin; B4, with 400 $\mu\text{g}/\text{ml}$ melatonin. Black and white combined bars indicate dark and light phases in LD. Black bars indicate DD.

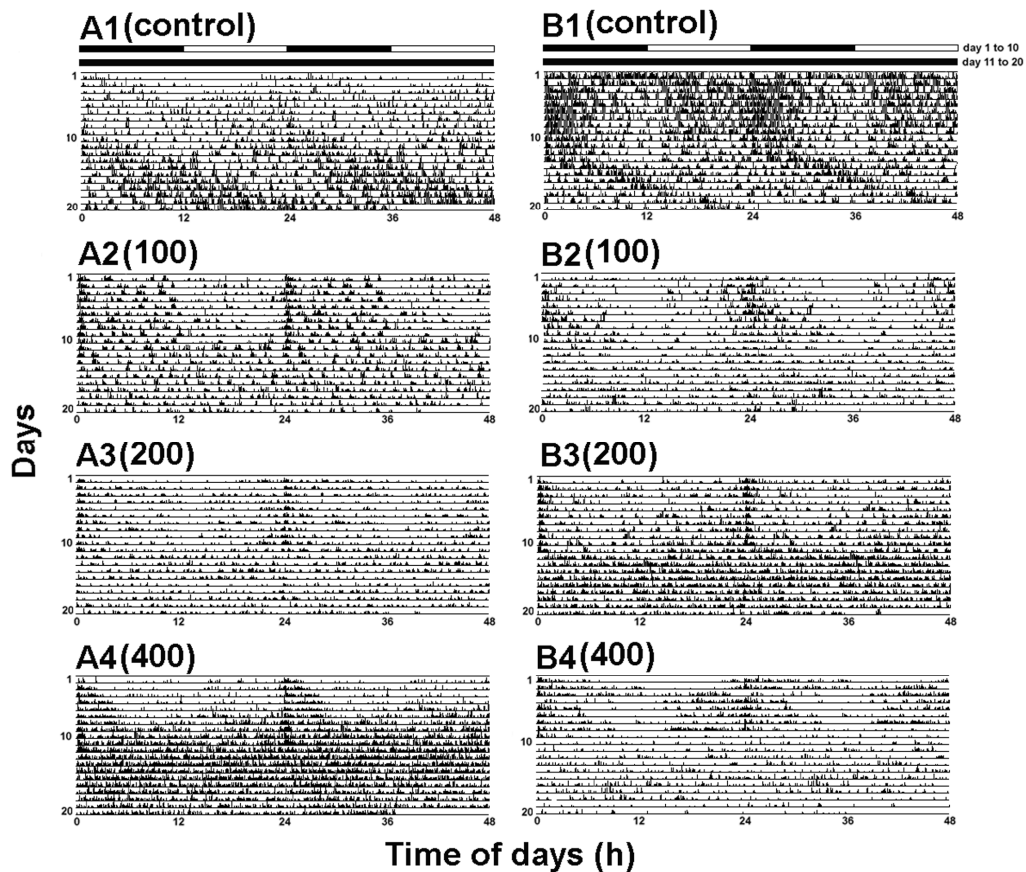


Fig. 3. Examples of locomotor activity patterns in males (A1–A4) and females (B1–B4) drinking caffeine and control. The Crickets were entrained under LD 12:12 for the first 10 days then transferred to DD for the second 10 days. A1, control; A2, with 100 $\mu\text{g/ml}$ caffeine; A3, with 200 $\mu\text{g/ml}$ caffeine; A4, with 400 $\mu\text{g/ml}$ caffeine; B1, control; B2, with 100 $\mu\text{g/ml}$ caffeine; B3, with 200 $\mu\text{g/ml}$ caffeine; B4, with 400 $\mu\text{g/ml}$ caffeine. Black and white combined bars indicate dark and light phases in LD. Black bars indicate DD.

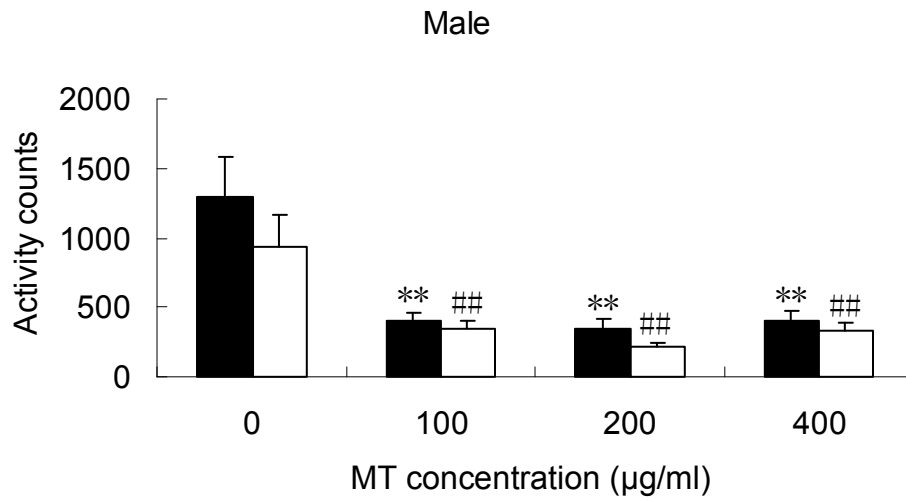


Fig. 4. Activity counts of males under LD 12:12 (white bars) and DD (black bars) of melatonin-treated groups and respective controls. **,## $p < 0.01$ under DD and LD 12:12, compared with respective control. MT, melatonin.

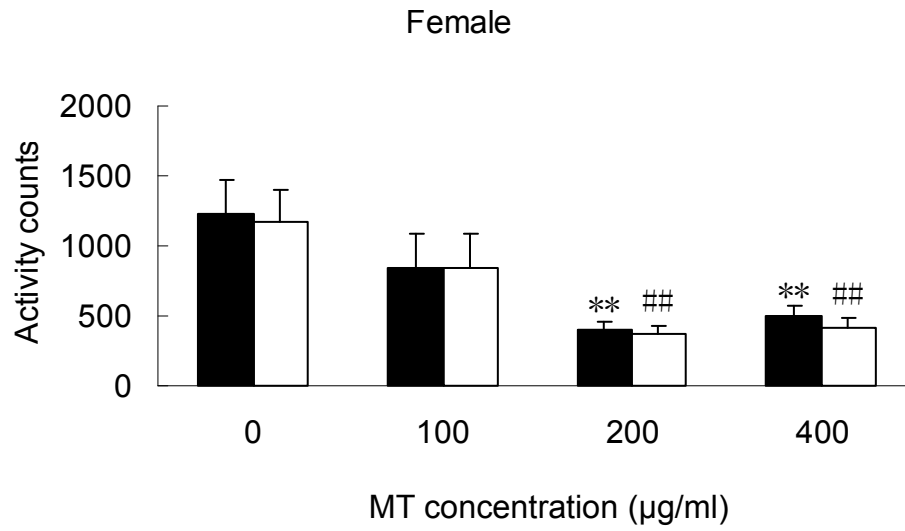


Fig. 5. Activity counts of females under LD 12:12 (white bars) and DD (black bars) of melatonin-treated groups and respective controls. **,## $p < 0.01$ under DD and LD 12:12, compared with respective control. MT, melatonin.

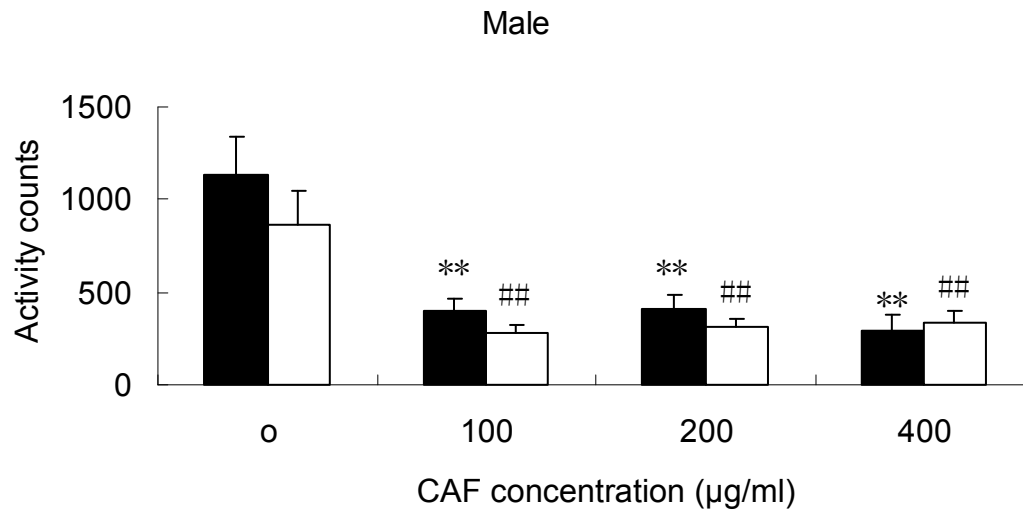


Fig. 6. Activity counts of males under LD 12:12 (white bars) and DD (black bars) of melatonin-treated groups and respective controls. **,## $p < 0.01$ under DD and LD 12:12, compared with respective control. CAF, caffeine.

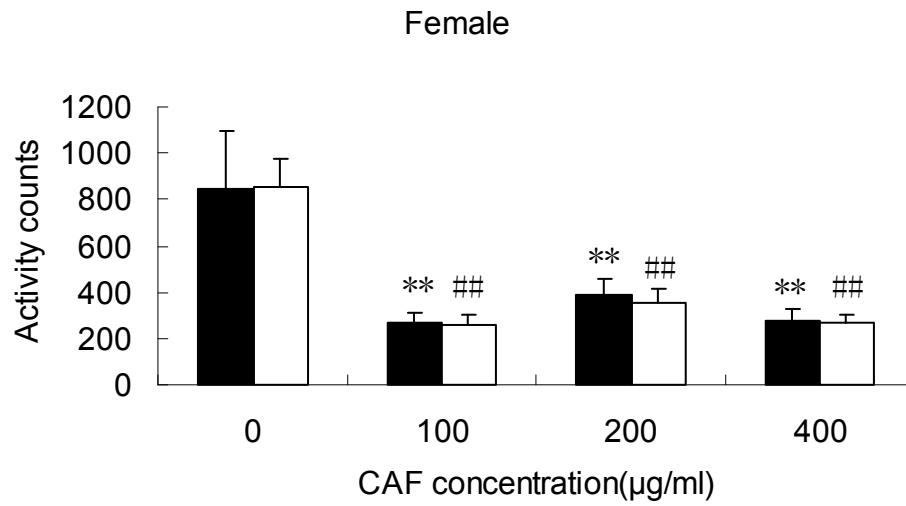


Fig. 7. Activity counts of females under LD 12:12 (white bars) and DD (black bars) of melatonin-treated groups and respective controls. **,### $p < 0.01$ under DD and LD 12:12, compared with respective control. CAF, caffeine.

Table 1 Effects of melatonin on activity counts and circadian rhythm parameters of males and females in LD 12:12.

Melatonin concentration ($\mu\text{g/ml}$)	Activity counts/ τ_{DD}	Acrophase (h)	Amplitude	T_{LD} (h)	N/T_{LD} (%)
♂					
0 (control)	928.19 ± 235.37^a	10.79 ± 2.50	0.30 ± 0.03	24.10 ± 0.09	60.77 ± 2.36^a
100	348.71 ± 53.72^b	8.29 ± 2.01	0.33 ± 0.03	24.09 ± 0.26	64.50 ± 3.24^{ab}
200	219.05 ± 25.44^b	11.62 ± 2.31	0.32 ± 0.03	25.00 ± 1.06	64.82 ± 2.86^{ab}
400	324.24 ± 66.60^b	9.88 ± 1.09	0.35 ± 0.03	24.10 ± 0.10	70.04 ± 3.40^b
♀					
0 (control)	1165.47 ± 237.46^a	13.06 ± 2.4	0.25 ± 0.03	24.26 ± 0.14	53.60 ± 2.64
100	848.62 ± 231.79^{ab}	10.98 ± 2.62	0.28 ± 0.03	24.03 ± 0.08	56.42 ± 3.17
200	377.06 ± 56.72^b	8.69 ± 2.63	0.32 ± 0.05	24.00 ± 0.25	59.45 ± 3.27
400	408.19 ± 73.06^b	12.76 ± 2.64	0.33 ± 0.05	24.16 ± 0.14	58.55 ± 4.12

^{a,b} $p < 0.05$, Fisher's LSD *post hoc* test following a one-way ANOVA for respective parameters.

Table 2 Effects of melatonin on activity counts and circadian rhythm parameters of males and females in DD.

	Melatonin concentration ($\mu\text{g/ml}$)	Activity counts/ τ_{DD}	Acrophase (h)	Amplitude	τ_{DD} (h)	N/T_{DD} (%)
♂	0 (control)	1290.38 $\pm$ 297.48 ^a	6.62 $\pm$ 2.02	0.22 $\pm$ 0.04	25.05 $\pm$ 0.54	55.75 $\pm$ 8.76
	100	409.83 $\pm$ 54.79 ^b	9.51 $\pm$ 1.98	0.24 $\pm$ 0.04	25.32 $\pm$ 0.32	55.32 $\pm$ 8.73
	200	342.29 $\pm$ 78.32 ^b	9.50 $\pm$ 1.98	0.18 $\pm$ 0.04	24.99 $\pm$ 0.43	53.52 $\pm$ 7.87
	400	404.19 $\pm$ 72.52 ^b	8.50 $\pm$ 1.78	0.24 $\pm$ 0.02	25.81 $\pm$ 0.37	53.92 $\pm$ 7.16
♀	0 (control)	1229.22 $\pm$ 241.83 ^a	8.92 $\pm$ 2.21	0.19 $\pm$ 0.04	24.51 $\pm$ 0.48	54.17 $\pm$ 1.95
	100	848.62 $\pm$ 231.79 ^{ab}	12.32 $\pm$ 2.12	0.16 $\pm$ 0.04	24.44 $\pm$ 0.65	53.95 $\pm$ 2.15
	200	399.87 $\pm$ 55.52 ^b	8.06 $\pm$ 2.35	0.19 $\pm$ 0.03	24.91 $\pm$ 0.48	56.75 $\pm$ 3.35
	400	495.77 $\pm$ 69.27 ^b	9.13 $\pm$ 2.46	0.24 $\pm$ 0.04	24.94 $\pm$ 0.59	50.74 $\pm$ 1.73

^{a,b} $p < 0.05$, Fisher's LSD *post hoc* test following a one-way ANOVA for respective parameters.

Table 3 Effects of caffeine on activity counts and circadian rhythm parameters of males and females entrained to LD 12:12.

	Caffeine concentration ($\mu\text{g/ml}$)	Activity counts/24 h	Acrophase (h)	Amplitude	τ_{LD} (h)	$N/T_{LD}(\%)$
♂	0 (control)	859.45 ± 191.13^a	11.33 ± 1.80	0.26 ± 0.02	24.47 ± 0.23	60.78 ± 2.37^a
	100	283.11 ± 39.14^b	8.69 ± 1.98	0.33 ± 0.03	24.16 ± 0.07	70.84 ± 2.99^b
	200	309.52 ± 48.41^b	7.89 ± 1.73	0.32 ± 0.03	24.18 ± 0.13	67.05 ± 3.81^{ab}
	400	333.19 ± 62.92^b	9.15 ± 2.22	0.27 ± 0.04	24.31 ± 0.24	63.92 ± 3.77^{ab}
♀	0 (control)	850.95 ± 125.39^a	15.56 ± 2.03^a	0.24 ± 0.02^a	24.48 ± 0.17^a	53.84 ± 2.83^a
	100	259.83 ± 46.62^b	14.67 ± 1.94^a	0.27 ± 0.03^{ab}	24.14 ± 0.12^b	53.15 ± 2.75^a
	200	349.95 ± 62.19^b	4.83 ± 1.67^b	0.33 ± 0.03^b	23.99 ± 0.02^b	62.11 ± 2.82^b
	400	264.29 ± 35.10^b	15.48 ± 2.36^a	0.28 ± 0.03^{ab}	24.13 ± 0.06^b	57.60 ± 1.52^{ab}

^{a, b} $p < 0.05$, Fisher's LSD *post hoc* test following a one-way ANOVA for respective parameters.

Table 4 Effects of caffeine on activity counts and circadian rhythm parameters of males and females in DD.

	Caffeine concentration ($\mu\text{g/ml}$)	Activity counts/ τ_{DD}	Acrophase (h)	Amplitude	τ_{DD} (h)	N/T_{DD} (%)
♂	0 (control)	1133.91 ± 201.83^a	18.10 ± 1.63	0.24 ± 0.03	25.65 ± 0.26	55.66 ± 1.66^a
	100	396.30 ± 63.16^b	14.85 ± 3.26	0.25 ± 0.04	25.19 ± 0.62	55.24 ± 1.97^a
	200	411.60 ± 71.13^b	11.15 ± 2.73	0.22 ± 0.04	25.68 ± 0.59	50.10 ± 1.41^b
	400	293.67 ± 81.61^b	16.14 ± 2.98	0.20 ± 0.04	25.13 ± 0.73	53.14 ± 1.57^{ab}
♀	0 (control)	842.91 ± 249.77^a	14.23 ± 2.77	0.19 ± 0.02	25.41 ± 0.52	57.18 ± 1.52
	100	267.44 ± 44.13^b	11.76 ± 2.93	0.15 ± 0.03	24.91 ± 0.78	52.67 ± 2.50
	200	390.22 ± 68.69^b	16.75 ± 2.64	0.17 ± 0.03	26.12 ± 0.55	52.78 ± 1.46
	400	275.42 ± 54.12^b	18.22 ± 2.11	0.16 ± 0.03	25.63 ± 0.56	53.53 ± 1.19

^{a,b} $p < 0.05$, Fisher's LSD *post hoc* test following a one-way ANOVA for respective parameters.

3.5 Discussion

Like other studies, melatonin also affected the circadian rhythm in our crickets. Firstly, it decreased the locomotor activity in both males and females of the crickets in LD 12:12 and DD significantly. This may be because melatonin suppresses the neuronal firing of the brain and the cAMP formation (Margarita, 2007; Cassone, 1990; Gaffori, 1985). Another agreeable result is that activity became more rhythmic because amplitude increased in both males and females in LD and DD. Melatonin modulates circadian rhythm by MT1 and MT2 melatonin receptors to synchronize the circadian rhythms (Margarita, 1988, 1998, 2007; Yamano et al., 2000).

On the other hand, activity phase advanced in LD 12:12 but delayed behind in DD, and τ_{DD} prolonged. The fraction of night activity increased while that of subjective night activity decreased. Study has showed that melatonin increased the night activity and decreased the daytime activity in nocturnal animals in the light-dark cycles (Cassone, 1990) and our study verifies this conclusion. In the constant dark, the fraction of night activity decreased, which may be the consequence of the prolonged free running period and the delayed acrophase. Without the restriction of light, melatonin affected the circadian rhythm during the whole cycle, not just focused on the night phase. The study has shown that injection melatonin in late night time delayed the activity phase of rats (Margarita, 1998). Besides, though we don't know when the tested crickets drank melatonin, we still propose that the drinking is different between LD and DD, because switching on and off resets the biological clock (Foster and Kreitzman, 2005).

Similarly, caffeine inhibited locomotor activity in our crickets at 100, 200, and 400 $\mu\text{g/ml}$. It advanced phase of activity; cut off light phase activity since total activity counts decreased but N/T_{LD} increased in LD; synchronizes the photoperiodic modulation of LD12:12, while decreased the amplitude in the constant dark; τ_{LD} became closer to the entrained 24h. This was additive to the effect of bright light, because bright light is believed to reduce motor activity in crickets (Koga et al., 2005). In DD, caffeine sank the circadian rhythm compared with the control, and the study showed that the constant darkness has little effect on the circadian system in cricket adults (Koga et al., 2005). Combined the effect on LD cycles, we suspect that caffeine is involved in the phototransduction pathway to the circadian pacemaker. On the other hand, it disordered the locomotor activity of DD, which indicates that caffeine had the antagonism with melatonin in the dark. Caffeine can alter the expression level of clock genes and change their amplitudes (Sherman et al., 2011), and the effects depend on different tissues. In the liver of mammals it has been approved that caffeine advanced the clock gene expression and altered their expression level. By the way, caffeine accumulates cAMP by blocking adenosine receptors (Fredholm et al., 1999). Change the clock gene expression can alter the intracellular cAMP levels and then shift the phase of clock gene expression (Sherman et al., 2011). Another explanation for the result is that caffeine can change the intracellular calcium signaling pathways by reducing the threshold of Caffeine-induced Ca^{2+} release, and then the amplitude comes down (Friel and Tsien, 1992). This can explain why the amplitude decreased in the constant dark, and hence proves that there are different mechanisms of caffeine

functions in the LD cycles and DD respectively. In addition, casein kinases and glycogen synthase kinases (GSK) modify period length in *Drosophila* and in mammals (Hendricks et al., 2000; Lin et al., 2002; Iitaka et al., 2005; Hirota et al., 2008; Tsuchiya et al., 2009), while caffeine may affect by control them indirectly.

In LD, the acrophase in females occurred in the early daytime, which was different from males. Interestingly, the 200 µg/ml caffeine treatment markedly advanced the phase such that acrophase appeared in the dark in females. But two-way ANOVA showed no significant difference in the effects between males and females (data not shown).

It's obvious that melatonin and caffeine modulate circadian rhythm in insects. Both of them decreased locomotor activity significantly in LD 12:12 and DD, which is similar to the effects on activity in LD 16:8 from **Chapter 2**. But extend was different, which shows the effects of two medicines depends on the light conditions. Melatonin synchronized the circadian rhythm no matter under what kinds of light conditions while caffeine showed two converse effects in LD and DD. It indicates that melatonin may be a rhythm modulator independent of light while caffeine takes function depending on light cycles. Our result proved again the theory that melatonin synchronizes the circadian rhythm and another theory that caffeine is a biphasic stimulant that does not always increase the locomotor activity but decreases it in the dose-dependent form. Finally, it's the first time clarifying the effect of caffeine on circadian rhythm in insects.

Chapter 4

Summary

We mainly studied the behavior of the cricket, *Gryllus vavius*, and tested its growth, metabolic rate in both nymphs and adults. Finally, we focused on the circadian rhythm in adults. In addition to the nature of above characters, we utilize melatonin and caffeine to test the pharmacology functions in this species.

In locust, *Locusta migratoria* melatonin is discovered at the first time. From then on, people started to study its functions and mechanism in various insects. One of its important functions is modulating the circadian rhythms of many kinds of physiological processes. Especially, it regulates sleep-waking cycle to move the activity phase and sleep phase. Melatonin decreases locomotor activity and improve the sleep to make the sleep-wake cycle more rhythmic. The secretion of melatonin mainly occurs at night and in the circadian rhythmic way in the brain. It is involved in circadian oscillator and found in the ocellar nerve in the insect. The newest molecular mechanism for its rhythm modulation is due to M1 and M2 receptor activation and the restriction of dopamine release. Recent studies show that melatonin also prolongs life span in animals by modulating the metabolism.

Caffeine is a biphasic and dose-dependent medicine, which increases the activity on lower concentration while decreases it on higher concentration, and also depends on different individuals. Although few studies reported that caffeine modulates the circadian rhythm in insects, it disorders the sleep and motion, lengthens circadian

rhythms in mice in the constant dark and delayed the phase of the mouse liver clock. It affected the clock genes expression and is tissue dependent.

Now, caffeine is believed as the antagonist of adenosine because it can combine A1 and A2 adenosine receptors to increase the neuron firing. Caffeine also is reported to prolong the life span of animals, and is dose-dependant as well.

The development of nymphs under long photoperiod is slower than that under short photoperiod. They acted less, and the metabolic rate was lower, but the body size was bigger, compared with those under short photoperiod. Therefore, there is a hypothesis that there is a chain connecting the metabolic rate, activity, growth period, and body size together. These characters are relative to each other and the change of one aspect can induce the others to change relatively.

Both melatonin and caffeine changed the above characters of physiology adhering to the above hypothesis. In nymphs, melatonin increased the activity level. Relatively, metabolic rate increased, growth duration of nymph stage decreased and body size became smaller. The result were the same under long and short photoperiods but more significant under short photoperiod. However, caffeine increased the activity level under long photoperiod but decreased it under short photoperiod. Consequently, metabolic rate increased, growth duration of nymph stage decreased and body size became smaller as well under long photoperiod, while it showed converse result under short photoperiod. The difference was significant between two photoperiods. It seems that photoperiods have some effects on the function of these two medicines, and affected caffeine more than melatonin.

In adults, two medicines kept the same trend in both sexes under LD 16:8, at 20 °C, which decreased the activity, and increased the growth period of adult stage. Relatively, metabolic rate decreased as well. The effect of melatonin was converse between nymphs and adults, which indicates that the sensitivities of nymphs and adults to melatonin are significantly different, or different rearing temperatures (30 °C in nymphs and 20 °C in adults) lead to the differences. The further study is needed to verify these two possibilities.

The circadian rhythm study proved that both melatonin and caffeine can modulate the circadian rhythm in crickets. They advance the acrophase of locomotor activity and also decreased the activity level both in LD 12:12 and DD, at 20 °C. The fraction of night activity increased in medicine drinking groups. The fraction of night activity was analyzed both in the video recording and locomotor activity chapters, the results are the same both in melatonin and caffeine. Compared with nymphs, the locomotor activity is more rhythmic in adults, which shows that males activate mainly during night, while females in the light.

During the constant dark, the free running period prolonged and acrophase lagged behind both in males and females after drinking melatonin. But the amplitude increased both in LD 12:12 and DD, which indicates that melatonin synchronizes the circadian rhythm in different light conditions. On the other hand, caffeine show obvious biphasic effects, which increased the amplitude in LD 12:12 while decreased it in the constant dark. Meanwhile, the free running period decreased in males but increased in females. Even though acrophase in DD advanced in males, the fraction of

night activity still decreased, same as acrophase delayed in females. It indicates that caffeine disordered the circadian rhythm in the constant dark. Obviously, melatonin and caffeine have different effects on circadian rhythm in the crickets, and this is the first time clarifying effects of caffeine on locomotor activity rhythm in insects, and proved the synchronization of melatonin and caffeine, and disorder function of caffeine.

The prolonged growth duration caused by medicine drinking indicates that the concentrations we chose are benefit to the age of the crickets. This is consistent with other studies that show the similar effects that these two medicines can extend the life span in mammals and humans. Moreover, both of them reduce the risk of Alzheimer's disease and cancer. Therefore, this species we used can be as a simple animal model to have some significance for humans. For the first time, we demonstrated the positive functions of caffeine on crickets and this line of research should be studied in more details.

For the future study, other concentrations of two medicines should also be tested to complete their dose-dependent functions in insects. The mechanisms causing these phenomena need to be clarified in the future to define the processes involved in determining how they affects human aging, health, and cancer. Considering of melatonin is suppressed by the light, the periodic application of melatonin by injection should be tested in the crickets.

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Appendix Papers

LI Xiaoting, TAKEDA Makio: Effects of photoperiods on activity level, developmental time, body size and metabolic rate in the nymph of cricket, *Gryllus varius* (Orthoptera: Gryllidae). Chinese Journal of Applied Entomology (in press)

LI Xiaoting, WATARI Yasuhiko, TAKEDA Makio: Effects of caffeine on locomotor activity, circadian rhythm, and longevity in the cricket, *Gryllus sp. nov.* #50

WEISSMAN. Biological Control (submitted)