Molecular analysis of the circadian clock system in the cricket, *Gryllus bimaculatus*

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Abstract

Circadian clocks are an important regulator of daily behavioral and physiological timing of animals. The molecular machinery of the circadian clock has been extensively studied in the fruit fly, *Drosophila melanogaster*. The oscillation of the *Drosophila* clock is thought to be generated by a molecular mechanism that is composed of transcriptional-translational autoregulatory feedback loops. Recent studies suggest that in other insect species, the clock mechanism may somewhat differ from that of *Drosophila*. The present study showed that knocking-down of clock-genes by RNAi persisted for a long period in two insect species, *Gryllus bimaculatus* and *Thermobia domestica*. The long-lasting effect of RNAi is convenient for chronobiological studies that require monitoring of physiological functions over long periods of time. Then, through molecular cloning, full length cDNA sequence of the clock gene *cycle* (*Gb'cyc*) was obtained. *Gb'cyc* was rhythmically expressed in the optic lobe with a peak similar to that of the *Gb'period* (*Gb'per*) and *Gb'timeless* (*Gb'tim*) around mid-night under LD. The *cyc* RNAi resulted in an oscillation of *Gb'Clock* (*Gb'Clk*) that was rather constitutively expressed in intact crickets. The circadian clock in *G. bimaculatus* might have a unique molecular oscillatory mechanism that has features of both *Drosophila* and mammalian clocks. The extra-optic lobe tissues also show rhythms of clock gene expression in nymphal and adult crickets. In nymphal crickets, the mRNA levels of clock genes were significantly lower than those in adults. Unlike in adults, the nymphal brain and mid-gut exhibited no rhythms of the clock gene expression in DD or when the optic lobes were bilaterally removed.

These results clearly provided fundamental information for utilizing RNAi technique in any long-running experiment and revealed that *Gb'cyc* is involved in the central machinery of the cricket circadian clock. More importantly, the results on measurement of *Gb'cyc* mRNA and *Gb'Clk* mRNA in *Gb'cyc* RNAi crickets suggest that the circadian clock in *G. bimaculatus* has a unique molecular oscillatory mechanism that has features of both *Drosophila* and mammalian clocks. Furthermore, the results of the clock gene expression outside the optic lobe suggest that the rhythms outside the optic lobe are weak in nymphs, become robust after the imaginal molt, and receive a control from the central clock.

List of abbreviations

ANOVA, analysis of variance
bHLH, basic helix-loop-helix
BCTR, BMAL1 C-terminal region
CK1, CASEIN KINASE1
CK2, CASEIN KINASE2
Clk, *Clock*
CLK, CLOCK
CODEHOP, consensus-degenerate hybrid oligonucleotide primers
cry, *cryptochrome*
CRY, CRYPTOCHROME
Cry2, *cryptochrome2*
CRY2, CRYPTOCHROME2
CT, circadian time
cwo, *clockwork orange*
cyc, *cycle*
CYC, CYCLE
DBT, DOUBLETIME
DD, constant darkness
dsRNA, double-stranded RNA
EAG, electroanntenographic
LD, light-dark cycle
MTs, Malpighian tubules
OLX, optic lobe removal
pdf, *pigment-dispersing factor*
PDF, pigment-dispersing factor
PDH, pigment-dispersing hormone
Pdp1ε, *PAR domain protein 1 ε*
PDP1ε, PAR DOMAIN PROTEIN 1ε
per, *period*
PER, PERIOD
RdRp, RNA-dependent RNA polymerase
RISC, RNA-induced silencing complex
RORα, retinoic acid receptor-related orphan receptor α
SCN, suprachiasmatic nuleus
SGG, SHAGGY

siRNA, small interfering RNA
TAG, terminal abdominal ganglion
tim, *timeless*
TIM, TIMELESS
vri, *vrille*
VRI, VRILLE
UTR, untranslated region
ZT, zeitgeber time

Chapter 1.

General introduction

1.1. Observations on circadian rhythms of insects

The majority of insects show daily activity cycles. They are nocturnal, diurnal or crepuscular. For example, cockroaches show almost nocturnal activity rhythms in light-dark (LD) conditions. Under constant conditions of light and temperature the locomotor activity rhythm has been shown to persist for several months in cockroaches (Roberts, 1960). The mosquito also shows daily rhythms in their flight activities that could be recorded by automatic devices, with which flight noise is amplified (Jones, 1964; Nayar and Sauerman, 1971). In LD 12:12 the mosquito *Anopheles gambiae* shows a bimodal activity pattern with an intense activity lasting 20 to 30 minutes following both light-off and light-on (Jones et al., 1966, 1967). The fruit fly also shows bimodal locomotor activity rhythms with peaks at around dawn and before dusk. When the flies are transferred to DD, they show free-running rhythms with a period of approximately 24 hr (Konopka and Benzer, 1971). The cricket *Gryllus bimaculatus* shows locomotor activity at night and this rhythm persists in isolated individuals in constant conditions in the laboratory (Tomioka and Chiba, 1982).

Once-in-a-lifetime events, such as adult emergence or larval hatching of insects, often occur at a particular time of day and the rhythm can be detected in a population consisting of individuals with different developmental stages. For example, the hatching of the cricket *G. bimaculatus* occurs rhythmically during the night, persisting in constant conditions (Tomioka et al., 1991). Egg hatching rhythms have been described in the corn borer *Diatraea grandiosella*

and the silk moth *Antheraea pernyi*. In both cases the rhythm free-ran in darkness (Takeda, 1983; Sauman et al., 1996).

These overt rhythms serve as an indirect marker for the state of the circadian clock. Daily locomotor activity rhythms are commonly observed in many insects and have been studied in holometabolous insects such as flies, beetles and moths as well as in hemimetabolous insects including cockroaches and crickets (Konopka and Benzer, 1971; Truman, 1972; Page and Barrett, 1989).

1.2. Localization of the circadian pacemaker in insects

The pacemakers regulating the circadian rhythm have been studied and localized to discrete regions of the brain. The brain of insect can be divided into two major areas, i.e. the optic lobe and the central brain. The optic lobes are paired bilateral structures that recieve input from the compound eyes. The visual imformation is then transmitted to the central brain.

Crickets and cockroaches were used to search for the clock location because their large size and ease of handling made them suitable experimental subjects. The optic lobes were found to be involved in generation of circadian oscillations in these insects. Removal of the two optic lobes resulted in a loss of locomotor activity rhythms in the cockroach *Leucophaea maderae* and the cricket *G. bimaculatus* (Page et al., 1977; Tomioka and Chiba, 1984, 1989). The fact suggests that the optic lobes either contain the circadian clock or are a part of the output pathway of the clock.

In contrast to cockroaches and crickets, results from flies and moths indicate importance of the central brain as the site of the relevant pacemaker. In

silk moths (*A. pernyi* and *H. cecropia*) extirpation of the optic lobes had no effect on the persistence of the flight activity rhythm, but removal of the cerebral lobes led to arrhythmicity (Truman, 1974). Locomotor activity rhythms of the house fly *Musca domestica* continued after surgical lesions of the optic lobes but disappeared after lesions of the cerebral brain (Helfrich et al., 1985). Similarly, circadian rhythms of locomotor activity in the fruit fly persisted in a variety of mutants with largely reduced optic lobes (Helfrich and Engelmann, 1987). The importance of the cerebral lobe in the rhythm generation has also been shown by a transplantation experiment in the fruit fly *D. melanogaster* (Handler and Konopka, 1979).

1.3. Molecular oscillatory mechanism of the *Drosophila* circadian clock

The molecular machinery of the circadian clock has been extensively studied in the fruit fly, *D. melanogaster*. The oscillation of the *Drosophila* clock is thought to be generated by a molecular mechanism that is composed of transcriptional-translational autoregulatory feedback loops (Dunlap, 1999). At least three interdependent feedback loops (Hardin, 2006; Sandrelli et al., 2008), in which so-called clock genes play a significant role, are thought to constitute the rhythm-generating machinery (Fig. 1-1).

One major loop is formed by *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), and *cycle* (*cyc*) (Hardin, 2006; Stanewsky, 2002). *per* and *tim* mRNA abundance oscillates in tandem with a maximum in the early night. Their transcription is activated by transcriptional activator CLK and CYC that are encoded by *Clk* (Allada et al., 1998) and *cyc* genes (Rutila et al., 1998). CLK and CYC proteins

contain a basic helix-loop-helix (bHLH) region allowing them to bind to a short DNA sequence called E-box in the promotor region of *per* and *tim* (Kyriacou and Rosato, 2000). PER and TIM proteins increase during the night and heterodimerize in the cytoplasm. The heterodimerization is mediated by PAS (stands for PER, ARNT and SINGLEMINDED) domains in PER. The PER/TIM heterodimer is then translocated to the nucleus to repress *per* and *tim* transcription through its inhibitory action to CLK/CYC (Williams and Sehgal, 2001). PER and TIM are posttranslationally regulated by DOUBLETIME (DBT), CASEIN KINASE 2 (CK2) and SHAGGY (SGG), and through this regulation their stability and the timing of nuclear transport are controlled (Akten et al., 2003; Martinek et al., 2001; Price et al., 1998).

Other genes involved as elements within the proposed loop include *vrille* (*vri*) and *PAR domain protein 1ε* (*Pdp1ε*) that regulate the rhythmic expression of *Clk*. The CLK-CYC heterodimer activates the transcription of *Pdp1ε* and *vri* during late day to early night. The *vri* mRNA is soon translated to its product protein VRI, which enters the nucleus, binds to a V/P-box in the promoter region of *Clk,* and inhibits its transcription. Thus the *Clk* mRNA is reduced during the night. PDP1ε is thought to bind to the V/P-box competitively with VRI and activates transcription of *Clk*. Thus, the *Clk* transcripts increase during the day, also leading to a subsequent increase of CLK protein (Cyran et al., 2003; Glossop et al., 2003).

The third loop includes *clockwork orange* (*cwo*), which is a transcriptional repressor belonging to the basic helix-loop-helix ORANGE family. *cwo* is rhythmically expressed to peak under the regulation by CLK-CYC and forms its

own negative feedback loop. CWO represses the expression of other clock genes, such as *per* and *tim*, through E-box elements (Kadener et al., 2007; Matsumoto et al., 2007).

1.4. The function of clock genes in other insects

The machinery of the *Drosophila* circadian clock has been understood in detail as described above. However, this hypothesis is not fully supported by recent studies using other insect species. For example, in the firebrat *Thermobia domestica*, *Td'Clk* transcripts show no rhythmic change both under light-dark cycles and constant darkness, in contrast to *Drosophila Clk* that shows rhythmic expression with antiphase against *timeless* (Kamae et al., 2010; Kamae et al., 2012). The honeybee *Apis mellifera* lacks *timeless* gene in its genome and has a mammalian-type *cryptochrome* (*cry2*) gene, leading to a conclusion that *A. mellifera* has a mammalian-type circadian clock (Rubin et al., 2006). In the silkmoth *Antheraea pernyi*, PER shows an oscillation in its abundance in the cytoplasm but not in the nucleus (Sauman and Reppert, 1996). In the monarch butterfly *Danaus plexippus*, *cry2* works together with *per* as a transcriptional repressor of the negative feedback loop similar to the mammalian clock (Zhu et al., 2008). Thus there might be a considerable diversification of the circadian clock in insects.

1.5. The circadian rhythms of peripheral tissues and their relationship to the central clock

Besides the central clock localized in the nervous system, there are

clocks in various peripheral tissues such as the compound eyes, antennae, prothoracic glands, Malpighian tubules (MTs), and testes. They are called ''peripheral clocks''. In *Drosophila*, many organs show circadian rhythms that maintain oscillations in an isolated and cultured condition (Giebultowicz and Hege, 1997; Plautz et al., 1997). Those include legs, proboscis, antennae, wings and MTs. These tissues can be entrained to light cycles and temperature cycles *in vitro* (Levine et al., 2002; Glaser and Stanewsky, 2005). Thus, they have a complete set of circadian clock including the entrainment mechanism. The central and peripheral relationship has been tested in MTs by transplanting them to an abdomen of host flies which had been entrained to the reversed LD cycle (Giebultowicz et al., 2000). The transplanted MTs maintained their original phase for several cycles, indicating that the clock in the MTs can oscillate independently of the central clock of the host. It is now generally accepted that in *Drosophila* the peripheral tissues have a tissue autonomous clock independent of the central clock and their proper phase is regulated by direct entrainment to environmental cycles.

In some cases, however, the peripheral clocks are apparently dependent on the central clock. In cockroaches, the antennal odor sensitivity rhythm measured by EAG is driven by the central clock, since it is lost when the optic tracts are bilaterally severed (Page and Koelling, 2003). The receptor cells in each sensillum, however, still maintain the sensitivity rhythm in those operated cockroaches (Saifullah and Page, 2009). Thus, the central clock organizes the temporal structure of the antenna. Severance of the optic nerves also prevents the ERG rhythms in the compound eye (Wills et al., 1985) unlike in *Drosophila*,

where rhythmic expression of PER protein in the compound eye persisted at least for a few days in disconnected mutant flies that lack neurons located in the lateral protocerebrum (Zerr et al., 1990). The molecular mechanisms and functions of the circadian clock vary in a tissue dependent and a species dependent manner (Fig. 1-2). This is probably because of different life-styles among insects.

1.6. The cricket behavior and neurobiology

The cricket displays elaborate behaviors that can be easily studied in the laboratory. Some of their behavioral patterns can be measured with high resolution, even in partially restrained animals. The cricket, *G. bimaculatus* has been used as a model insect in behavior and neurobiology fields for a long time. For example, the cricket has been used to investigate a fascinating acoustic communication system involved in calling, courtship, and fighting behaviors (Hedwig, 2006; Loher and Dambach, 1989), a discriminatory olfactory learning system (Matsumoto and Mizunami, 2000), and embryonic development and appendage regeneration (Nakamura et al., 2010; Mito et al., 2002). There have been many studies of the acoustic, tactile, visual and mating behavior of crickets (Kutsch and Huber, 1989; Schildberger et al., 1989; Gnatzy and Hustert, 1989; Honegger and Campan, 1989; Matsumoto and Sakai, 2000a,b). The activities of cerebral neurons have been studied during phonotactic orientation (Böhm and Schildberger, 1992; Staudacher and Schildberger, 1998) and of neurons in the terminal abdominal ganglion during escape responses evoked by air currents (Hörner, 1992; Kohstall-Schnell and Gras, 1994).

1.7. The objective of this study

The cricket, *G. bimaculatus* shows punctual diurnal activity in nymphal stage but becomes nocturnal after imaginal molt. Its central circadian clock has been localized in the optic lobe and the functions of clock genes could be investigated by molecular method such as RNA interference (Tomioka and Abdelsalam, 2004; Danbara et al., 2010; Moriyama et al., 2008 and 2012). Thus, the cricket is the best insect model for studying the phase setting controlled by the circadian clock at a molecular level.

In this study, I addressed the following three issues: (1) fundamental information for utilizing RNAi technique for effective knock-down of cricket's gene; (2) molecular oscillatory mechanism of the circadian clock in the cricket; (3) relationships between central and peripheral clocks in the circadian organization. The clock gene *cycle* was cloned from the cricket *G. bimaculatus* and its function in circadian rhythm generation was analyzed. Since it is very difficult to isolate mutants in the cricket, RNA interference mediated gene silencing was used as a major tool to dissect the molecular mechanism. I first attempted to determine the optimal concentration of double-stranded RNA (dsRNA) for systemic RNAi and the period of persistence of the RNAi effect in two insect species, the cricket *G. bimaculatus* and the firebrat *T. domestica*. To investigate the central and peripheral relationships in the circadian organization of nymphal and adult crickets, circadian rhythms of tissues outside the optic lobes were examined by measuring mRNA levels of clock genes in nymphal and adult crickets before and after the optic lobe removal. The goals of this study were twofold: establishing the fundamental knowledge

of the clock in the cricket and development of the cricket *G. bimaculatus* as a good model insect for molecular study of the circadian clock system.

References

Akten, B., Jauch, E., Genova, G.K., Kim, E.Y., Edery, I., Raabe, T., Jackson, F.R. (2003) A role for CK2 in the *Drosophila* circadian oscillator. Nat Neurosci 6:251-257.

Allada, R., White, N.E., So, W.V., Hall, J.C., Rosbash, M. (1998) A mutant *Drosophila* homolog of mammalian Clock disrupts circadian rhythms and transcription of period and timeless. Cell 93, 791–804.

Böhm, H., Schildberger, K. (1992) Brain neurones involved in the control of walking in the cricket *Gryllus bimaculatus*. J. Exp. Biol. 166, 113–130.

Cyran, S.A., Buchsbaum, A.M., Reddy, K.L., Lin, M.C., Glossop, N.R.J., Hardin, P.E., Young, M.W., Stori, R.V., Blau, J. (2003) *vrille*, *Pdp1* and *dClock* form a second feedback loop in the *Drosophila* circadian clock. Cell 112:329-341.

Danbara, Y., Sakamoto, T., Uryu, O., Tomioka, K. (2010) RNA interference of *timeless* gene does not disrupt circadian locomotor rhythms in the cricket *Gryllus bimaculatus*. J Insect Physiol 56:1738-1745.

Dunlap, J.C. (1999) Molecular bases for circadian clocks. Cell 96:271-290.

Giebultowicz, J.M., Hege, D.M. (1997) Circadian clock in Malpighian tubules. Nature 386:664.

Giebultowicz, J.M., Riemann, J.G., Raina, A.K., Ridgway, R.L. (1989) Circadian system controlling release of sperm in the insect testes. Science 245: 1098–1100.

Giebultowicz, J.W., Stanewsky, R., Hall, J.C., Hege, D.M. (2000) Transplanted *Drosophila* excretory tubules maintain circadian clock cycling out of phase with the host. Current Biology 10: 107–110.

Gnatzy, W., Hustert, R. (1989) Mechanoreceptors in behavior. In Cricket Behavior and Neuroethology (ed. F. Huber, T. E. Moore and W. Loher), pp. 198–226. New York: Cornell University Press.

Glaser, F.T., Stanewsky, R. (2005) Temperature synchronization of the *Drosophila* circadian clock. Curr Biol 15:1352–1363

Glossop, N.R., Houl, J.H., Zheng, H., Ng, F.S., Dudek, S.M., Hardin, P.E. (2003) VRILLE feeds back to control circadian transcription of *Clock* in the *Drosophila* circadian oscillator. Neuron 37:249-261.

Hardin, P.E. (2006) Essential and expendable features of the circadian timekeeping mechanism. Curr Opin Neurobiol 16:686–692.

Hedwig, B. (2006) Pulses, patterns and paths: neurobiology of acoustic behaviour in crickets. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 192:677-689.

Helfrich, C., Cymborowski, B., Engelmann, W. (1985) Circadian activity rhythm of the house fly continues after optic tract severance and lobectomy. Chronobiology International 2:19-32.

Helfrich, C., Engelmann, W. (1987) Evidences for circadian rhythmicity in the per0 mutant of *Drosophila melanogaster*. Z Naturforsch C. 42:1335-1338.

ind-evoked escape running of the cricket *Gryllus bimaculatus*. II. Neurophysiological analysis. J. Exp. Biol. 171: 215–245.

Honneger, H.W., Campan, R. (1989) Vision and visually guided behavior. In Cricket Behavior and Neuroethology (ed. F. Huber, T. E. Moore and W. Loher), pp. 147–177. New York: Cornell University Press.

Ito, C., Goto, S.G., Shiga, S., Tomioka, K., Numata, H. (2008) Peripheral circadian clock for the cuticle deposition rhythm in *Drosophila melanogaster*. Proc Natl Acad Sci U S A America 105, 8446–8451.

Jones, M.D.R. (1964) The automatic recording of mosquito activity. J Insect Physiol 10:343-351.

Jones, M.D.R., Ford, M.G., Gillett, J.D. (1966) Light-on and light-off effects on the circadian flight activity in the mosquito *Anopheles gambiae*. Nature, Lond 211:871-872.

Jones, M.D.R., Hill, M. and Hope, A.M. (1967) The circadian flight activity of the mosquito *Anopheles gambiae*: phase setting by the light regime. J exp Biol 47:503-511.

Kadener, S., Stoleru, D., McDonald, M., Nawathean, P., Rosbash, M. (2007) *Clockwork orange* is a transcriptional repressor and a new *Drosophila* circadian pacemaker component. Genes Dev 21:1675-1686.

Kamae, Y., Tanaka, F., Tomioka, K. (2010) Molecular cloning and functional analysis of the clock genes, clock and cycle, in the firebrat *Thermobia domestica*. J Insect Physiol 56: 1291–1299.

Kamae, Y., Tomioka, K. (2012) timeless is an essential component of the circadian clock in a primitive insect, the firebrat *Thermobia domestica*. J Biol Rhythms 27:126-134.

Kohstall-Schnell, D., Gras, H. (1994) Activity of giant interneurones and other wind-sensitive elements of the terminal ganglion in the walking cricket. J Exp Biol. 193: 157–181.

Konopka, R.J. and Benzer, S. (1971) Clock mutants of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 68:2112-2116.

Krishnan, B., Levine, J.D., Lynch. M.K., Dowse, H.B., Funes, P., Hall, J.C., Hardin, P.E., Dryer, S.E. (2001) A new role for cryptochrome in a *Drosophila* circadian oscillator. Nature 411, 313–317.

Kutsch, W., Huber, F. (1989) Neural basis of song production. In Cricket Behavior and Neuroethology (ed. F. Huber, T. E. Moore and W. Loher), pp. 262–309. New York: Cornell University Press.

Kyriacou, C.P., Rosato, E. (2000) Squaring up the E-box. J Biol Rhythms 15:483-90.

Levine, J.D., Funes, P., Dowse, H.B., Hall, J.C. (2002) Advanced analysis of a cryptochrome mutation's effects on the robustness and phase of molecular cycles in isolated peripheral tissues of *Drosophila*. BMC Neurosci 3:5

Loher, W., Dambach, M. (1989) Reproductive Behavior. In Cricket Behavior and Neuroethology (ed. F. Huber, T. E. Moore and W. Loher), pp. 43–82. New York: Cornell University Press.

Martinek, S., Inonog, S., Manoukian, A.S., Young, M.W. (2001) A role for the segment polarity gene shaggy/GSK-3 in the *Drosophila* circadian clock. Cell 105:769-779.

Matsumoto, A., Ukai-Tadenuma, M., Yamada, R.G., Houl, J., Uno, K.D., Kasukawa, T., Dauwalder, B., Itoh, T.Q., Takahashi, K., Ueda, R., Hardin, P.E., Tanimura, T., Ueda, H.R. (2007) A functional genomics strategy reveals *clockwork orange* as a transcriptional regulator in the *Drosophila* circadian clock. Genes Dev 21:1687-1700.

Matsumoto, Y., Sakai, M. (2000a). Brain control of mating behaviour in the male cricket *Gryllus bimaculatus* DeGeer: the center for inhibition of copulation actions. J. Insect Physiol. 46: 527–538.

Matsumoto, Y., Sakai. M. (2000b). Brain control of mating behaviour in the male cricket *Gryllus bimaculatus* DeGeer: brain neurons responsible for inhibition of copulation actions. J. Insect Physiol. 46: 539–552.

Matsumoto, Y., Mizunami, M. (2000) Olfactory learning in the cricket *Gryllus bimaculatus*. J Exp Biol. 203:2581-2588.

Mito, T., Inoue, Y., Kimura, S., Miyawaki, K., Niwa, N., Shinmyo, Y., Ohuchi, H., Noji, S. (2002) Involvement of hedgehog, wingless, and dpp in the initiation of proximodistal axis formation during theregeneration of insect legs, a verification of the modified boundary model. Mech Dev. 114:27-35.

Moriyama, Y., Sakamoto, T., Karpova, S.G., Matsumoto, A., Noji, S., Tomioka, K. (2008) RNA interference of the clock gene period disrupts circadian rhythms in the cricket *Gryllus bimaculatus*. Journal of Biological Rhythms 23: 308–318.

Moriyama, Y., Kamae, Y., Uryu, O., Tomioka, K. (2012) *Gb'Clock* Is Expressed in the Optic Lobe and Required for the Circadian Clock in the Cricket *Gryllus bimaculatus*. J Biol Rhythms 27:467-477.

Nakamura, T., Yoshizaki, M., Ogawa, S., Okamoto, H., Shinmyo, Y., Bando, T., Ohuchi, H., Noji, S., Mito, T. (2010) Imaging of transgenic cricket embryos reveals cell movements consistent with a syncytial patterning mechanism. Curr Biol. 20:1641-1647.

Nayar, J. K., Sauerman, D. M. (1971) The effect of light regimes on the circadian rhythm of flight activity in the mosqito *Aedes taeniorhynchus*. J exp Biol 54:745-756.

Page, T.L., Caldarola, P.C., Pittendrigh, C.S. (1977)
Mutual entrainment of bilaterally distributed circadian pacemaker. Proc Natl Acad Sci U S A. 74:1277-1281.

Page, T.L. (1982) Transplantation of the cockroach circadian pacemaker. Science 216:73-75.

Page, T.L., Barrett, R.K. (1989) Effects of light on circadian pacemaker development. II. Responses to light. J Comp Phisiol A. 165:51-59.

Page, T.L., Koelling, E. (2003) Circadian rhythm in olfactory response in the antennae controlled by the optic lobe in the cockroach. J Insect Physiol 49: 697–707.

Plautz, J.D., Kaneko, M., Hall, J.C., Kay, S.A. (1997) Independent photoreceptive

circadian clocks throughout *Drosophila*. Science 278: 1632–1635.

Price, J.L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B., Young, M.W. (1998) double-time is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. Cell 94:83-95.

Roberts, S.K. de F. (1960) Circadian activity in cockroaches. I. The free-running rhythm in steady-state. J cell Comp Physiol 55:99-110

Rubin, E.B., Shemesh, Y., Cohen, M., Elgavish, S., Robertson, H.M., Bloch, G. (2006) Molecular and phylogenetic analyses reveal mammalian-like clockwork in the honeybee (*Apis mellifera*) and shed new light on the molecular evolution of the circadian clock. Genome Res 16: 1352-1365.

Rutila, J.E., Suri, V., Le, M., So, W.V., Rosbash, M., Hall, JC. (1998) CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. Cell 93:805-814.

Sauman, I. and Reppert, S.M. (1996) Circadian clock neurons in the silkmoth *Antheraea pernyi*: Novel mechanisms of period protein regulation. Neuron 17:889-900.

Sandrelli, F., Costa, R., Kyriacou, C.P., Rosato, E. (2008) Comparative analysis of circadian clock genes in insects. Insect Mol Biol 17:447–463

Saifullah, ASM., Page, T.L. (2009) Circadian regulation of olfactory receptor neurons in the cockroach antenna. J Biol Rhythms 24:144–152

Schildberger, K., Huber, F., Wohker, W. (1989) Central auditory pathway: Neural correlates of phonotactic behavior. In Cricket Behavior and Neuroethology (ed. F. Huber, T. E. Moore and W. Loher), pp. 423–458. New York: Cornell University Press.

Staudacher, E., Schildberger, K. (1998) Gating of sensory responses of descending brain neurones during walking in crickets. J. Exp. Biol. 201, 559–572.

Stanewsky, R. (2002) Clock mechanisms in *Drosophila*. Cell Tissue Res 309:11–26

Takeda, M. (1983) Ontogeny of the circadian system governing ecdysial rhythms in a holometabolous insect, *Diatraea grandiosella* (Pyralidae). Physiol Entomol 8: 321-331.

Tomioka, K., Chiba, Y. (1982) Persistence of circadian ERG rhythms in the cricket with optic tract severed. Naturwissenschaften 69: 355–356.

Tomioka, K., Chiba, Y. (1984) Effects of Nymphal Stage Optic Nerve Severance or Optic Lobe Removal on the Circadian Locomotor Rhythm of the Cricket, *Gryllus bimaculatus*. Zool Sci 1:375-382.

Tomioka, K. and Chiba, Y. (1989) Photoperiodic entrainment of locomotor activity in crickets (*Gryllus bimaculatus*) lacking the optic lobe pacemaker. J Insect Physiol 35:827-835.

Tomioka, K., Wakatsuki, T., Shimono, K., Chiba, Y. (1991) Circadian control of hatching in the cricket, *Gryllus bimaculatus*. J Insect Physiol 37:365-371.

Tomioka, K. and Abdelsalam, S. (2004) Circadian organization in hemimetabolous insects. Zool Sci 21:1153-1162.

Truman, J.W. (1972) Physiology of insect rhythms. I. Circadian organization of the encorine events underlying the moulting cycle of larval tobacco hornworms. J exp Biol 57:805-820.

Truman, J.W. (1974) Physiology of insect rhythms IV. Role of the brain in the regulation of the flight rhythm of the giant silkmoths. J Comp Physiol 95:281-296.

Williams, J.A., Sehgal. A. (2001) Molecular components of the circadian system in *Drosophila*. Annu Rev Physiol 63:729-55.

Wills, S.A., Page, T.L., Colwell, C.S. (1985) Circadian rhythms in the electroretinogram of the cockroach. J Biol Rhythms 1: 25–37.

Wiedenmann, G., Lukat, R., Weber, F. (1986) Cyclic layer deposition in the cockroach endocuticle: a circadian rhythm? J Insect Physiol 32:1019–1027.

Zerr, D.M., Hall, J.C., Rosbash, M., Siwicki, K.K. (1990) Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of *Drosophila*. J Neurosci 10:2749–2762

Zhu, H., Sauman, I., Yuan, Q., Casselman, A., Emery-Le, M., Emery, P., Reppert, S.M. (2008) Cryptochromes define a novel circadian clock mechanism in monarch butterflies that may underlie sun compass navigation. PLoS Biol 6:138-155.

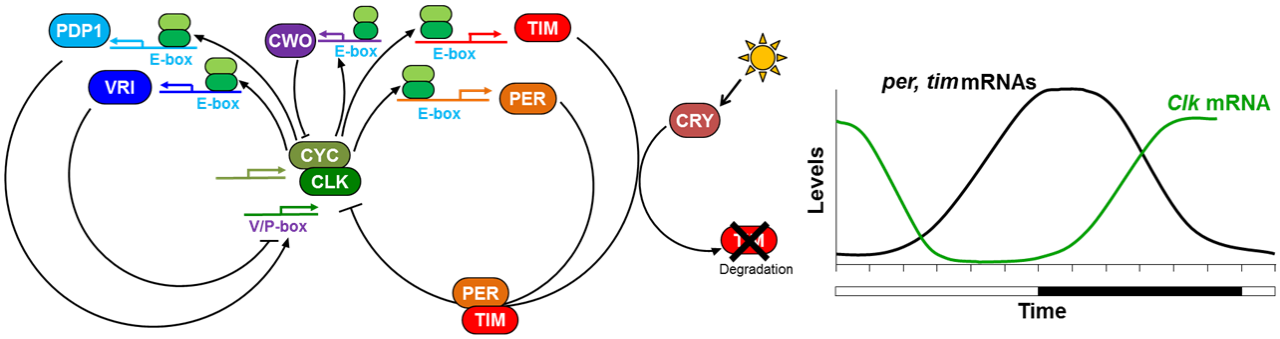

Fig. 1-1. The molecular oscillatory mechanism of the *Drosophila* clock.
CLK and CYC form a heterodimer that promotes transcription of *per*, *tim*, *vri* and *Pdp1ε* through E-box during late day to early night. Thus levels of *per* and *tim* transcripts begin to rise at late day. During the late day, translated TIM proteins are degraded by light-activated CRY: a light-dependent reset mechanism of the clock. As PER and TIM levels increase during midnight, the proteins form a stable complex that is capable of moving into the nucleus, the PER-TIM complex represses transcription of *per* and *tim* through inhibitory action to CLK-CYC. In the late night, phosphorylated PER no longer bind with TIM, and TIM is degraded by the proteasome system. The CLK-CYC heterodimer is thus released from suppression to reactivate *per* and *tim* transcription, starting the next cycle. Meanwhile, VRI accumulates during early night and represses *Clk* transcription through its binding to VRI/PDP1-box (V/P-box). Later accumulating PDP1ε activates *Clk* transcription by competitive binding to V/P-box with VRI, leading to a rhythmic expression of CLK with a peak at early day. *cwo* is rhythmically expressed to peak under the regulation by dCLK-CYC and forms its own negative feed-back loop. White and black bars indicate light and dark phase, respectively.

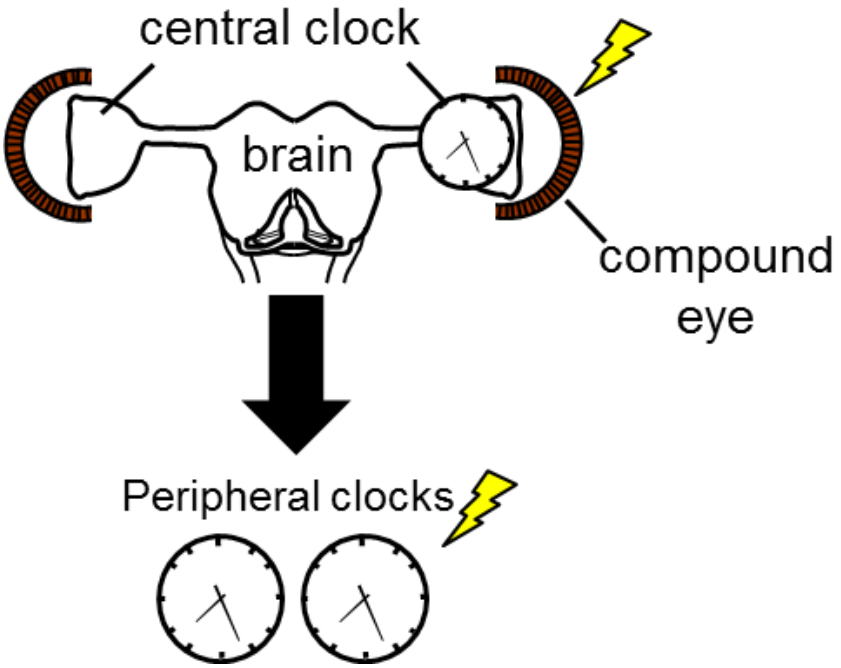

Fig. 1-2. The Central and peripheral clock structure

The relationship of the central and peripheral clocks can be assumed. All clocks individually perceive light directly or indirectly through photoreceptors, e.g., the compound eye for the optic lobe clock (top, right) and the thoracic photoreceptive elements for the epithelial cuticle deposition clock in *Drosophila*, so that they synchronize each other under light–dark cycle. Some peripheral tissues of *Drosophila* have tissue autonomous clocks independent of the central clock and their proper phase is regulated by direct entrainment to environmental cycles. The molecular mechanisms and functions of the circadian clock vary in a tissue dependent and a species dependent manner.

Chapter 2.

Long-term effect of systemic RNA interference on circadian clock genes in hemimetabolous insects

2.1. Abstract

RNA interference (RNAi) strategy, which enables gene-specific knock-down of transcripts, has been spread across a wide area of insect studies for investigating gene function without regard to model and non-model insects. This technique is of particular benefit to promote molecular studies on non-model insects. However, the optimal conditions for RNAi are still not well understood because of its variable efficiency depending on the species, target genes, and experimental conditions. To apply RNAi technique to long-running experiments such as chronobiological studies, the effects of RNAi have to persist throughout the experiment. In this study, it was attempted to determine the optimal concentration of double-stranded RNA (dsRNA) for systemic RNAi and its effective period in two different insect species, the cricket *Gryllus bimaculatus* and the firebrat *Thermobia domestica*. In both species, higher concentrations of dsRNA principally yielded a more efficient knock-down of mRNA levels of tested clock genes, although the effect depended on the gene and the species. Surprisingly, the effect of the RNAi reached its maximum effect 1–2 weeks and 1 month after the injection of dsRNA in the crickets and the firebrats, respectively, suggesting a slow but long-term effect of RNAi. This study provides fundamental information for utilizing RNAi technique in any long-running experiment.

2.2. Introduction

RNA interference (RNAi) is a highly conserved mechanism in eukaryotes that protects organisms from invasive/parasitic nucleic acids such

as viruses and transposons (Belles, 2010; Lozano et al., 2012). Eukaryotic cells can be stimulated by double-stranded RNA (dsRNA) and thereupon destroy mRNAs that share sequences with the dsRNA, resulting in the inhibition of virus/transposon activities. To achieve this process, the introduced long dsRNA is first digested by Dicer, a dsRNA-specific endonuclease, to short double stranded RNA fragments called small interfering RNAs (siRNA;~20–23 nucleotides; reviewed in e.g. Burand and Hunter (2012) and Hutvagner and Zamore (2002)). Next, the siRNAs are unwound, separated in single strands, and loaded into the RNA-induced silencing complex (RISC). With guide of the single strand siRNAs (called guide-strands), RISC targets mRNA that has a complementary sequence and cleaves the mRNA or interrupts its transcription, eventually leading to silencing of target mRNA. After the discovery of RNAi by Fire et al. (1998) in *Caenorhabditis elegans* (*C. elegans*), RNAi has been used as a tool to study gene function. In insects, dsRNA is usually introduced either by injection of dsRNA into the body or a tissue (systemic RNAi) or by feeding (Huvenne and Smagghe, 2010). Both methods need a mechanism by which cells take up exogenous dsRNA to initiate RNAi. In *C. elegans*, it is known that a transmembrane protein, SID-1, plays an important role in this dsRNA uptake (Winston et al., 2002), whereas the function of insect orthologs of the sid-1 gene have been under dispute (reviewed in Huvenne and Smagghe (2010)). The best known model insect, *D. melanogaster*, is less successful in systemic RNAi and lacks *sid* orthologs (e.g. Miller et al., 2008; Roignant et al., 2003), while Saleh et al. (2006) showed that scavenger receptors, which play a role in endocytosis, are involved in dsRNA uptake in both *Drosophila melanogaster* and *C. elegans*.

The most common way to introduce dsRNA into non-model insects is injection of dsRNA into their bodies because the feeding method is less effective and requires much higher concentrations of dsRNA. Surprisingly, it appears that the effect of the systemic RNAi can persist for a long time (Tomioka et al., 2009). When RNAi was performed for the first time in the cricket *G. bimaculatus* for the *period* (*Gb'per*) gene, which is a circadian clock gene in animals, locomotor rhythms were almost completely disrupted for more than 50 days after ds*period* RNA (ds*per* RNA) injection (Moriyama et al., 2008). Similar long-lasting effects of RNAi were observed for other clock genes (Danbara et al., 2010; Moriyama et al., 2012) and also in other insect species, such as the German cockroach *Blattella germanica* and the firebrat *Thermobia domestica* (Lee et al., 2009; Kamae et al., 2010; Kamae and Tomioka, 2012). These studies suggested that a single injection of dsRNA is sufficient for gene silencing over a long period, while almost nothing is known about a time effect on systemic RNAi in insects.

Before investigating the functions of many genes by systemic RNAi in the future, it is worthwhile to determine optimal conditions for RNAi and its effective period. In this study, it was thus tested different concentrations of dsRNA for several clock genes and investigated the long-term effect of RNAi in the cricket and the firebrat. This study found that the best gene knock-down occurs 1–2 weeks (crickets) and 1 month (firebrats) after the dsRNA injection, whereas the dsRNA level rapidly decreases before the strongest effect of RNAi is observed. This study will discuss this mismatch between the RNAi effect and the level of exogenous dsRNA.

2.3. Materials and methods

Animals

Adult male crickets, *G. bimaculatus*, and adult male and female firebrats, *T. domestica*, were used. They were obtained from a laboratory colony maintained under a light-dark (LD) cycle of 12 h of light and 12 h of darkness at a constant temperature of 25 ºC (crickets) and 30 ºC (firebrats).

Systemic RNAi

Target gene dsRNAs were synthesized using Megascript High Yield Transcription kit (Ambion, Austin, TX) as previously described (Kamae et al., 2010; Kamae and Tomioka, 2012; Moriyama et al., 2008, 2012; Chapter 3). The specificity of the dsRNAs was already confirmed in the previous studies using non-specific dsRNA, thereby demonstrating that injection of the non-specific dsRNA does not have any effect on the target RNA levels. The obtained dsRNA was adjusted to a final concentration of 10–20 μM with ultrapure water (Invitrogen, Carlsbad, CA), depending on the viscosity of the dsRNA solution. The dsRNA solution was stored at -80 ºC until use. 1520 nl (*Gb'per*), 760 nl (other cricket genes) and 70 nl (all firebrat genes) of dsRNA solutions were injected into the abdomen of the crickets and the firebrats with the nanoliter injector (WPI, Sarasota, FL) after anesthesia with CO_2 at Zeitgeber time 7–10 (ZT; ZT0 = lights-on, ZT12 = lights-off).

Quantitative real-time PCR

mRNA and dsRNA levels were measured using quantitative real-time RT-PCR (qPCR) as described previously (Kamae and Tomioka, 2012; Moriyama

et al., 2012). Total RNAs were extracted with TRIzol (Invitrogen) from 6 optic lobes (crickets) and from 5 whole bodies (firebrats), and the obtained RNA was treated with DNase I to remove contaminating genomic DNA. Reverse transcription was conducted with random 6mers and Primescript™ RT reagent kit (Takara, Otsu, Japan). Universal SYBR Green Master (Roche Diagnostics, Tokyo, Japan) containing SYBR green was used for DNA polymerase. All primers used in this study are listed in Table 2-1. qPCR was performed using the Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA). All values were normalized to the values for *Gb'rpl18a* (GenBank/EMBL/DDBJ Accession No. DC448653) and *Td'rp49* (GenBank/EMBL/DDBJ Accession No. AB550830), which are housekeeping genes serving as an internal control for the cricket and the firebrat, respectively. Results of 3–4 independent experiments were pooled to calculate the mean ± SEM. Statistics was performed using Tukey–Kramer multiple comparison test.

Behavior analysis

Locomotor activities were recorded as described previously (Moriyama et al., 2008). Briefly, adult crickets were individually housed in transparent plastic boxes (18 × 9 × 4.5 cm) with a plastic plate that seesaws by the movement of the cricket. The number of movements of the seesaw plate was recorded by a magnetic sensor during consecutive 6 min intervals. Food and water were provided ad libitum. The recording apparatus was placed in an incubator (MIR-153, Sanyo Biomedica, Osaka, Japan) to control temperature and light. Temperature in the incubator was kept at 25 ^{o}C throughout the recoding. The dsRNA injection was performed at ZT7~10 on the first day of the

recording. The control crickets were administrated non-specific dsRNA that derives from a coral gene (*DsRed2*) as described in Moriyama et al., (2012). The raw data were displayed as double-plotted actograms to judge activity patterns using ActogramJ (http://actogramj.neurofly.de/) (Schmid et al., 2011).

2.4. Results

RNAi efficiency with different dsRNA concentrations

In previous studies, 10 and 20 μM concentrations of dsRNAs were routinely used for injection into *G. bimaculatus* (e.g. Moriyama et al., 2008). These concentrations were determined by finding a practicable viscosity of the dsRNA solution which does not interfere with injection using the nanoliter injector. To investigate sufficient amounts of dsRNA required for RNAi in the cricket, I diluted the dsRNA solutions for the clock genes, *Gb'per* (GenBank/ EMBL/DDBJ Accession No. BAG48878), *Clock* (*Gb'Clk*; GenBank/EMBL/ DDBJ Accession No. AB738083) and *cycle* (*Gb'cyc*; GenBank/EMBL/ DDBJ Accession No. AB762416) which have been used in previous studies (Moriyama et al., 2008, 2012; Chapter 3) and injected these into the adult crickets. The optic lobes, in which the cricket central circadian clock resides, were sampled at the time-point of the maximum expression of each gene: at ZT6 for *Gb'Clk* and ZT18 for *Gb'per* and *Gb'cyc* under LD 12:12 and total RNA was extracted to measure mRNA levels of each gene with qPCR. In all tested clock genes, the highest concentrations of dsRNA caused the most efficient gene knock-down (Fig. 2-1A), while the sensitivity to dsRNAs depended on the gene. DsRNA concentrations of 1 and 2 μM were, for instance, sufficient to reduce mRNA

levels of *Gb'per* and *Gb'Clk* to significant levels, respectively, whereas a higher concentration (20 μM) was required for *Gb'cyc* to achieve a significant reduction.

The same approach was taken for another insect species, the firebrat *Thermobia domestica*. Since the *Td'per* gene has not successfully been cloned in the firebrat, the *timeless* gene was examined (*Td'tim*; GenBank/EMBL/DDBJ Accession No. AB644410) instead of *Td'per*, whereas *Td'Clk* (GenBank/EMBL/DDBJ Accession No. AB550828) and *Td'cyc* (GenBank/EMBL/DDBJ Accession No. AB550829) were the same as in the cricket experiment. In the firebrat, all clock genes were significantly knocked-down even with the lowest concentration (1 μM) of dsRNA (Fig. 2-1B). Moreover, the RNAi effect was not further enhanced with increasing concentration, suggesting that 1 μM of dsRNA is already sufficient for the gene knock-down in firebrats. This study took the different body size of the cricket and the firebrat into account and re-calculated the injected dsRNA amount per weight (Fig. 2-1). For each of the three firebrat genes, 1 μM of dsRNA solutions corresponded to 1.12–1.21 μg/g. This amount was much less than the 5–10 μM (2.33–4.33 μg/g) dsRNA concentrations used in the crickets. Nevertheless, the efficiency of the RNAi response was not higher in the crickets, suggesting that RNAi sensitivity also depends on the species. Particularly the *cyc* gene seems to be more difficult to knock-down in the cricket than in the firebrat. The *Gb'cyc* knock-down was improved step by step with increasing concentrations of ds*cyc* RNA (Fig. 2-1A). Thus, the amount of dsRNA may be increased for a more efficient knock-down.

The effective period of the systemic RNAi

Next, this study examined how long this RNAi effect lasts. The *Clk* gene was selected for this experiment because this gene showed an efficient RNAi response in both species. 20 μM of ds*Clk* RNA solution was injected into the adult crickets and the time-course profile of *Gb'Clk* mRNA levels was examined over 2 weeks. Compared to the untreated crickets, *Gb'Clk* mRNA levels were barely reduced 24 h after the injection ($P < 0.05$), and the reduction at 48 and 96 h was not significantly different (Fig. 2-2A). Surprisingly, the most efficient knock-down for *Gb'Clk* mRNA occurred 1–2 weeks after the injection. Thus, the systemic RNAi knocks down gene expression within a short period to a certain extent, but a longer period is required to obtain the most efficient RNAi effect.

The same experiment was repeated in the firebrats and injected 10 μM of ds*Clk* RNA. This species has an advantage for this kind of experiment because their lifespan is longer than those crickets. Therefore, the time-course observation was extended up to 2 months. On average, the firebrats molted ca. two times within this 2 months. Like in the crickets, *Td'Clk* mRNA levels were not significantly reduced until 96 h (4 days), and 1 week was required to detect a significant reduction ($P < 0.05$; Fig. 2-2B), although the reduction in this experiment was somehow less compared to the previous experiment (see Fig. 2-1B). The most efficient reduction was observed 1 month after the dsRNA injection ($P < 0.01$), and the mRNA level recovered to control levels in the next month. Thus, the effect of RNAi is time-limited.

The slow RNAi effect was also observed at the behavioral level in the crickets. After the injection of ds*Clk* RNA solution, the crickets exhibited a

free-running rhythm with a period of ca. 24 h for first 4 days in constant dark conditions (DD), and then the free-running period started to lengthen as was observed in a previous study (Fig. 2-3B; Moriyama et al., 2012). The control crickets did not change the period during the experiment (Fig. 2-3A).

Persistence of the administered dsRNA

It was wondered whether the injected dsRNA survived in the body for such a long period as more than 2 weeks. Therefore, this study designed a pair of primers that recognizes ds*Clk* cDNA: this primer set binds to a sequence within the ds*Clk* cDNA. Since discrimination between ds*Clk* RNA and endogenous *Clk* mRNA was difficult in this protocol, *Clk* mRNA levels of untreated crickets and untreated firebrats were used as a control. 24 h after the injection, qPCR detected about six times higher expression compared to the average *Gb'Clk* mRNA expression levels in the untreated crickets (Fig. 2-4A), thereby demonstrating that the primers detected ds*Clk* RNA together with the endogenous *Clk* mRNA. However, this high level of RNA rapidly declined and was no longer significantly different from the untreated animals 1 week after the injection, although additionally the reduced level of endogenous *Clk* mRNA had to be taken into account which is caused by RNAi. In the firebrats, *Td'Clk* RNA levels (ds*Clk* RNA and endogenous *Clk* mRNA) were about 80 times higher than the intact levels 24 h after the ds*Clk* RNA injection, but the level declined rapidly and 1 week after the injection there was already no significant difference in the RNA levels between the untreated and the treated animals (Fig. 2-4B). Altogether, this dsRNA degradation kinetics suggests that dsRNA would mostly be digested within 1 week, whereas the RNAi effect occurs later,

showing that the long-term effect of RNAi is not simply due to a long lifespan of the injected dsRNA within the insect body.

2.5. Discussion

Systemic RNAi is a powerful tool to investigate gene function in non-model insects. However, the range of its application is not fully understood in insect science. Inspired by a great publication studying the experimental conditions for RNAi conducted by a group of Lepidoptera researchers (Terenius et al., 2011), it was decided to investigate the experimental conditions of RNAi for the experimental insects. The main aim in this study was to identify the most efficient period of the RNAi effect in the cricket and the firebrat because the long-lasting effects of RNAi on locomotor rhythms were previously discovered (reviewed in Tomioka et al., (2009)). This was confirmed at the level of mRNA and it was shown that the clock-gene knock-down persists for a long period in both insect species (Fig. 4-2). This long-lasting RNAi effect is fortunately convenient for the chronobiological studies which require monitoring of physiological functions over long periods of time.

A big question remaining to be answered is why the most efficient gene knock-down was observed when the level of dsRNA was low. One hypothesis is that small fragments of dsRNA (siRNAs) derived from dsRNA digested by the Dicer endonuclease have a long lifespan in the body and that siRNAs work as the guide-strands for RISC over a month, at least in the case of the firebrats. This dsRNA digestion and the accumulation of siRNA may take a long time, leading to the slow RNAi effect. An alternative explanation would be that these

insects might have a mechanism to endogenously amplify the siRNAs by an RNA polymerase. In *C. elegans* and plants, exogenous siRNA is used as a template for RNA-dependent RNA polymerase (RdRp) that produces secondary siRNAs, thereby enhancing and extending the RNAi effect (Pak and Fire, 2007; Sijen et al., 2007; reviewed in Rother and Meister (2011)). This multiplicative effect is called ''transitive property'' of the RNAi and is apparently important for the RNAi effect in *C. elegans* (reviewed in May and Plasterk (2005)). Unfortunately, a homolog of RdRp has not been identified in insect genomes so far (reviewed in Rother and Meister (2011)). However, a recent study in *D. melanogaster* has shown that the *Drosophila* elongator subunit 1 gene, *Delp1*, has RdRp activity and that this gene is well conserved in all eukaryotes (Lipardi and Paterson, 2009). Therefore, it is possible that crickets and firebrats also possess a homolog of the *elp1* gene and can replicate siRNA by its RdRp activity, leading to the longlasting RNAi effects. However, it must be noted here that the RNAi effect does not last forever, as the knock-down of *Td'Clk* could not be observed in the firebrats after 2 months (Fig. 2-2B).

During this study, it was realized that tests of different concentrations of dsRNA and observation of the time-course profile of gene knock-down provide useful information. Since extremely high concentrations of dsRNA may cause non-specific off-target effect, it may be necessary to determine sufficient amounts of dsRNA for the injection. The experiment investigating the time-course profile helps to estimate the right time after the dsRNA administration to conduct the experiment. The injection of dsRNA into the maternal body to perform parental RNAi may also be a possibility to control

the long-term effect of RNAi if a researcher needs to test the phenotype in an early developmental stage (Bucher et al., 2002; Liu and Kaufman, 2004; Mito et al., 2008; Ronco et al., 2008; Sakamoto et al., 2009).

In summary, this study provides fundamental information for conducting systemic RNAi for long-running experiments in insects. Since RNAi became a common tool to investigate the function of clock genes in insect species (e.g. Ikeno et al., 2010; Kotwica et al., 2009; Tobback et al., 2012), it is interesting to study interspecies differences in RNAi efficiency for clock genes.

References

Belles, X. (2010) Beyond *Drosophila*: RNAi *in vivo* and functional genomics in insects. Annual Review of Entomology 55, 111–128.

Bucher, G., Scholten, J., Klingler, M. (2002) Parental RNAi in Tribolium (Coleoptera). Current Biology: CB 12, R85–R86.

Burand, J.P., Hunter, W.B. (2012) RNAi: future in insect management. Journal of Invertebrate Pathology.

Danbara, Y., Sakamoto, T., Uryu, O., Tomioka, K. (2010) RNA interference of timeless gene does not disrupt circadian locomotor rhythms in the cricket *Gryllus bimaculatus*. Journal of Insect Physiology 56, 1738–1745.

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

Hutvagner, G., Zamore, P.D. (2002) RNAi: nature abhors a double-strand. Current Opinion in Genetics & Development 12, 225–232.

Huvenne, H., Smagghe, G. (2010) Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. Journal of Insect Physiology 56, 227–235.

Ikeno, T., Tanaka, S.I., Numata, H., Goto, S.G. (2010) Photoperiodic diapause under the control of circadian clock genes in an insect. BMC Biology 8, 116.

Kamae, Y., Tanaka, F., Tomioka, K. (2010) Molecular cloning and functional

analysis of the clock genes, Clock and cycle, in the firebrat *Thermobia domestica*. Journal of Insect Physiology 56, 1291–1299.

Kamae, Y., Tomioka, K. (2012) Timeless is an essential component of the circadian clock in a primitive insect, the firebrat *Thermobia domestica*. Journal of Biological Rhythms 27, 126–134.

Kotwica, J., Bebas, P., Gvakharia, B.O., Giebultowicz, J.M. (2009) RNA interference of the period gene affects the rhythm of sperm release in moths. Journal of Biological Rhythms 24, 25–34.

Lee, C.M., Su, M.T., Lee, H.J. (2009) Pigment dispersing factor: an output regulator of the circadian clock in the German cockroach. Journal of Biological Rhythms 24, 35–43.

Lipardi, C., Paterson, B.M. (2009) Identification of an RNA-dependent RNA polymerase in *Drosophila* involved in RNAi and transposon suppression. Proceedings of the National Academy of Sciences of the United States of America 106, 15645–15650.

Liu, P.Z., Kaufman, T.C. (2004) Kruppel is a gap gene in the intermediate germband insect *Oncopeltus fasciatus* and is required for development of both blastoderm and germ band-derived segments. Development 131, 4567–4579.

Lozano, J., Gomez-Orte, E., Lee, H.J., Belles, X. (2012) Super-induction of Dicer-2 expression by alien double-stranded RNAs: an evolutionary ancient response to viral infection? Development Genes and Evolution 222, 229–235.

May, R.C., Plasterk, R.H. (2005) RNA interference spreading in *C. elegans*. Methods in Enzymology 392, 308–315.

Miller, S.C., Brown, S.J., Tomoyasu, Y. (2008) Larval RNAi in *Drosophila*? Development Genes and Evolution 218, 505–510.

Mito, T., Ronco, M., Uda, T., Nakamura, T., Ohuchi, H., Noji, S. (2008) Divergent and conserved roles of extradenticle in body segmentation and appendage formation, respectively, in the cricket *Gryllus bimaculatus*. Developmental Biology 313, 67–79.

Moriyama, Y., Kamae, Y., Uryu, O., Tomioka, K. (2012) *Gb'Clock* is expressed in the optic lobe and is required for the circadian clock in the cricket *Gryllus bimaculatus*. Journal of Biological Rhythms 27, 467–477.

Moriyama, Y., Sakamoto, T., Karpova, S.G., Matsumoto, A., Noji, S., Tomioka, K. (2008) RNA interference of the clock gene period disrupts circadian

rhythms in the cricket *Gryllus bimaculatus*. Journal of Biological Rhythms 23, 308–318.

Pak, J., Fire, A. (2007) Distinct populations of primary and secondary effectors during RNAi in C. elegans. Science 315, 241–244.

Roignant, J.Y., Carre, C., Mugat, B., Szymczak, D., Lepesant, J.A., Antoniewski, C. (2003) Absence of transitive and systemic pathways allows cell-specific and isoformspecific RNAi in *Drosophila.* RNA 9, 299–308.

Ronco, M., Uda, T., Mito, T., Minelli, A., Noji, S., Klingler, M. (2008) Antenna and all gnathal appendages are similarly transformed by homothorax knock-down in the cricket *Gryllus bimaculatus*. Developmental Biology 313, 80–92.

Rother, S., Meister, G. (2011) Small RNAs derived from longer non-coding RNAs. Biochimie 93, 1905–1915.

Sakamoto, T., Uryu, O., Tomioka, K. (2009) The clock gene period plays an essential role in photoperiodic control of nymphal development in the cricket *Modicogryllus siamensis*. Journal of Biological Rhythms 24, 379–390.

Saleh, M.C., van Rij, R.P., Hekele, A., Gillis, A., Foley, E., O'Farrell, P.H., Andino, R. (2006) The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. Nature Cell Biology 8, 793–802.

Schmid, B., Helfrich-Forster, C., Yoshii, T. (2011) A new ImageJ plug-in ''ActogramJ'' for chronobiological analyses. Journal of Biological Rhythms 26, 464–467.

Sijen, T., Steiner, F.A., Thijssen, K.L., Plasterk, R.H. (2007) Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. Science 315, 244–247.

Terenius, O., Papanicolaou, A., Garbutt, J.S., Eleftherianos, I., Huvenne, H., Kanginakudru, S., Albrechtsen, M., An, C., Aymeric, J.L., Barthel, A., Bebas, P., Bitra, K., Bravo, A., Chevalier, F., Collinge, D.P., Crava, C.M., de Maagd, R.A., Duvic, B., Erlandson, M., Faye, I., Felfoldi, G., Fujiwara, H., Futahashi, R., Gandhe, A.S., Gatehouse, H.S., Gatehouse, L.N., Giebultowicz, J.M., Gomez, I., Grimmelikhuijzen, C.J., Groot, A.T., Hauser, F., Heckel, D.G., Hegedus, D.D., Hrycaj, S., Huang, L., Hull, J.J., Iatrou, K., Iga, M., Kanost, M.R., Kotwica, J., Li, C., Li, J., Liu, J., Lundmark, M., Matsumoto, S., Meyering-Vos, M., Millichap, P.J., Monteiro, A., Mrinal, N., Niimi, T., Nowara, D., Ohnishi, A., Oostra, V.,

Ozaki, K., Papakonstantinou, M., Popadic, A., Rajam, M.V., Saenko, S., Simpson, R.M., Soberon, M., Strand, M.R., Tomita, S., Toprak, U., Wang, P., Wee, C.W., Whyard, S., Zhang, W., Nagaraju, J., Ffrench-Constant, R.H., Herrero, S., Gordon, K., Swevers, L., Smagghe, G. (2011) RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design. Journal of Insect Physiology 57, 231–245.

Tobback, J., Vuerinckx, K., Boerjan, B., Huybrechts, R. (2012) RNA interference mortality points to noncircadian functions for the clock gene in the desert locust Schistocerca gregaria. Insect Molecular Biology 21, 369–381.

Tomioka, K., Sakamoto, T., Moriyama, T. (2009) RNA interference is a powerful tool for chronobiological study in the cricket. Sleep and Biological Rhythms 7, 144–151.

Uryu, O., Karpova, S.G., Tomioka, K. (2013) The clock gene cycle plays an important role in the circadian clock of the cricket *Gryllus bimaculatus*. J Insect Physiol 59: 697-704

Winston, W.M., Molodowitch, C., Hunter, C.P. (2002) Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1. Science 295, 2456–2459.

Table 2-1. Primer sequences used for qPCR

	Forward	Reverse
G. *bimaculatus*		
per	5′-AAGCAAGCAAGCATCCTCAT-3′	5′-CTGAGAAAGGAGGCCACAAG-3′
Clk	5′-AATGACCGTAGTCGAGAAAGTGAAG-3′	5′-TTGCGATGATTGAGGTTGTTG-3′
Clk for dsRNA[a]	5′-GCATCACTTCTCTTCTGGGTCA-3′	5′-CATACTGCGCACCATCAACAC-3′
cyc	5′- GGCCGAAGCTCATAAAGTGG -3′	5′- AACCGCACAAAGGAACCATC -3′
rpl18a	5′-GCTCCGGATTACATCGTTGC-3′	5′-GCCAAATGCCGAAGTTCTTG-3′
T. domestica		
tim	5′-TACAAGCCAGGTCCATCACA-3′	5′-TCAAGCGTCAATTCAGCATC-3′
Clk	5′-ATCGCAAGGGTCTGGAAGTG-3′	5′-GGAAAACTCGCCAAGACAGG-3′
Clk for dsRNA[a]	5′-CCGCAAGTGGATAAGGCAAG-3′	5′-CCCAGTTCCCACGAAAACTAA-3′
cyc	5′-CGTGTAATCTGTCGTGTTTGGTG-3′	5′-GAATCGTCCGCCTTTCCTC-3′
rp49	5′-AGTCCGAAGGCGGTTTAAGG-3′	5′-TACAGCGTGTGCGATCTCTG-3′

[a] primer sets used for detecting exogenous dsRNA.

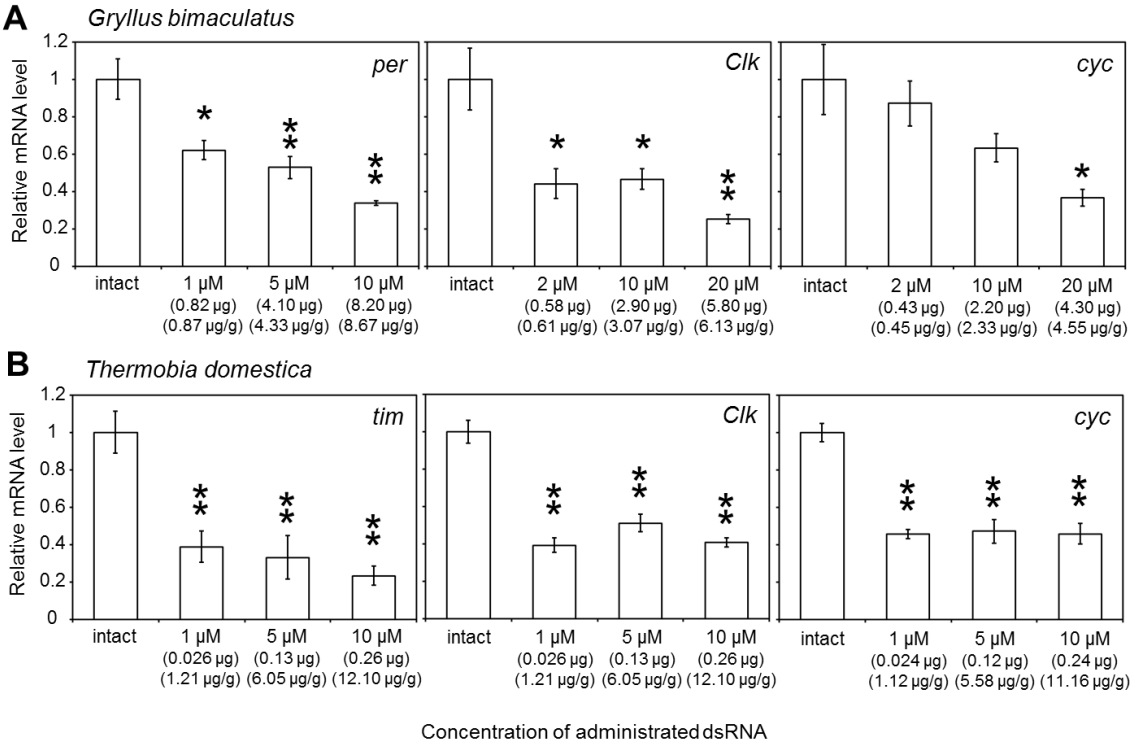

Fig. 2-1. Dose-dependent effects of dsRNAs on clock genes in *G. bimaculatus* (A) and in *T. domestica* (B).

Three different concentrations of dsRNA were tested for *per*, *Clk* and *cyc* genes for the crickets, and for *tim*, *Clk* and *cyc* genes for the firebrats. In both species dsRNA was injected at ZT7~10. (A) Total RNA was extracted from 6 optic-lobes of adult crickets that were sampled at ZT6 for *Clk* and ZT18 for *per* and *cyc* under LD 12:12 conditions 7 days after the dsRNA injection. Mean (± SEM) mRNA levels were normalized to the average values of untreated, non-injected animals. The intact level is set to 1.0. For ds*per* RNA 1520 nl and for ds*Clk* and ds*cyc* RNA 760 nl were injected into the abdomens. The concentration and amount of dsRNA are indicated by mol concentration (M), the total amount of injected dsRNA (in μg) and the total amount of dsRNA by the average weight of the cricket (in μg/g). The average weight of 20 adult crickets was 946 mg. (B) Total RNA was extracted from 5 whole firebrats that were sampled at ZT10 under LD12:12 conditions 7 days after the dsRNA injection. The average weight of 20 adult firebrats was 21.5 mg. Asterisks indicate significant differences from the level of untreated animals (* $P < 0.05$; ** $P < 0.01$, Tukey–Kramer multiple comparison test).

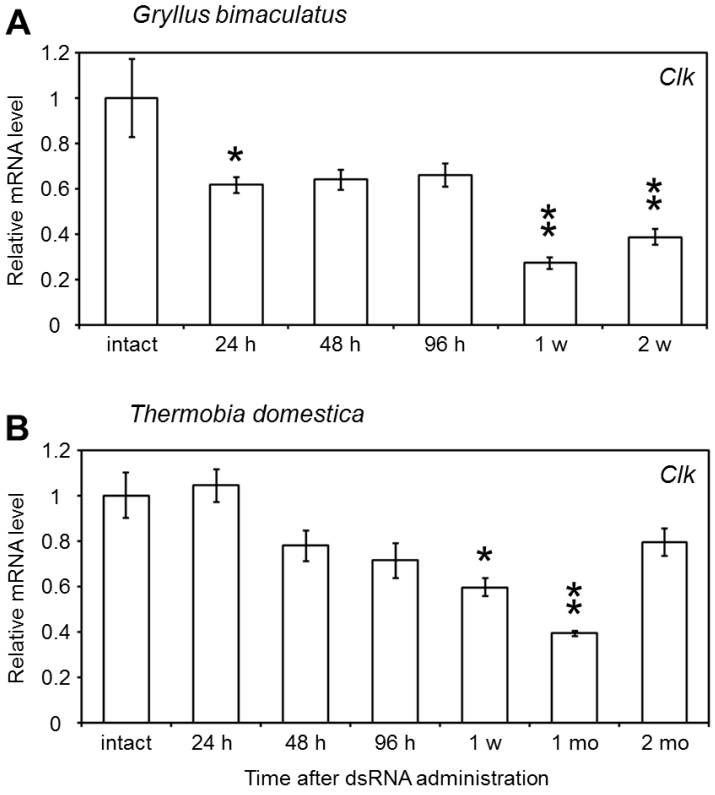

Fig. 2-2. Time-course profiles of the RNAi effect on *Clk* gene expression in *G. bimaculatus* and in *T. domestica*.

20μM (crickets) and 10 μM (firebrats) of dsRNA were used for injection. Total RNA was extracted from 6 optic-lobes of injected crickets and 5 bodies of injected firebrats that were sampled at ZT10 in LD 12:12 conditions. (A) *Clk* mRNA levels in crickets were examined 24, 48, 96 h, 1 week (w), and 2 w after the dsRNA injection. The maximum knock-down occurred 1–2 weeks after the dsRNA injection. (B) *Clk* mRNA levels of firebrats were examined 24, 48, 96 h, 1 w, 1 month (mo), and 2 mo after the dsRNA injection. The maximum knockdown occurred 1 month after the dsRNA injection. The data are shown as mean ± SEM after normalization to the mean value of the untreated animals. The intact level is set to 1.0. Asterisks indicate significant differences from the level of untreated animals (* $P < 0.05$; ** $P < 0.01$, Tukey–Kramer multiple comparison test).

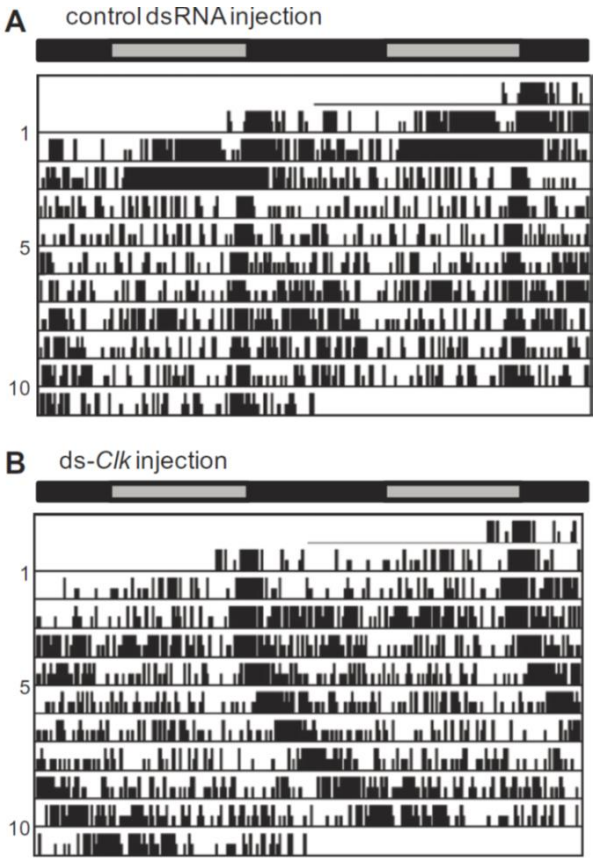

Fig. 2-3. Representative actograms of individual crickets under constant dark conditions.

The bars above the actograms indicate previous light conditions where the crickets were kept before starting the experiment. 20 μM of dsRNA for nonspecific RNA (A; *DsRed2* derived from a coral gene) and for *Clk* gene (B) were injected to the crickets right before the start of recording. At ZT12 light was off followed by a constant darkness for 10 days. The control crickets exhibited a free-running rhythm with a period length of about 24 h, whereas the *Clk* RNAi crickets changed their period after 4 days.

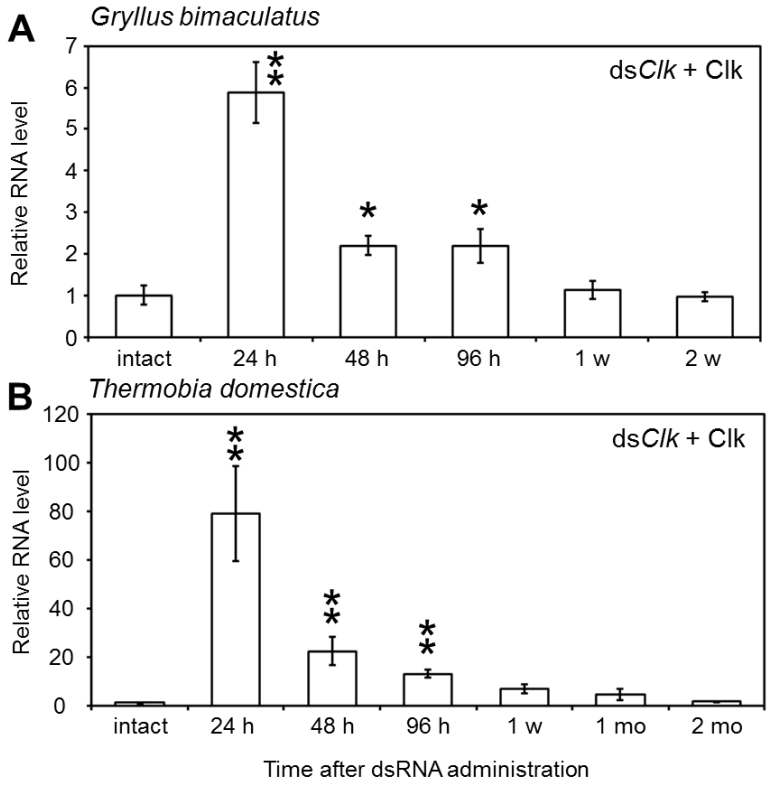

Fig. 2-4. The dsRNA levels in crickets (A) and firebrats (B) were investigated in a time series using a pair of primers that binds within the dsRNA sequence and also within the endogenous *Clk*.

The data are shown as mean ± SEM after normalization to the mean value of the untreated animals. The intact level is set to 1.0. The value of the untreated animals virtually indicates the mRNA level of the endogenous *Clk* gene expression. Asterisks indicate significant differences from the level of untreated animals (* $P < 0.05$; ** $P < 0.01$, Tukey–Kramer multiple comparison test). See Fig. 2- 3 for more details.

Chapter 3.

The clock gene *cycle* plays an important role in the circadian clock of the cricket *Gryllus bimaculatus*

3.1. Abstract

To dissect the molecular oscillatory mechanism of the circadian clock in the cricket *Gryllus bimaculatus*, a cDNA of the clock gene *cycle* (*Gb'cyc*) was cloned and its structure and function were analyzed. *Gb'cyc* contains four functional domains, i.e. bHLH, PAS-A, PAS-B and BCTR domains, and is expressed rhythmically in light dark cycles, peaking at mid night. The RNA interference (RNAi) of *Clock* (*Gb'Clk*) and *period* (*Gb'per*) reduced the *Gb'cyc* mRNA levels and abolished the rhythmic expression, suggesting that the rhythmic expression of *Gb'cyc* is regulated by a mechanism including *Gb'Clk* and *Gb'per*. These features are more similar to those of mammalian orthologue of *cyc* (*Bmal1*) than those of *Drosophila cyc*. A single treatment with double-stranded RNA (dsRNA) of *Gb'cyc* effectively knocked down the *Gb'cyc* mRNA level and abolished its rhythmic expression. The *cyc* RNAi failed to disrupt the locomotor rhythm, but lengthened its free-running period in constant darkness (DD). It is thus likely that *Gb'cyc* is involved in the circadian clock machinery of the cricket. The *cyc* RNAi crickets showed a rhythmic expression of *Gb'per* and *timeless* (*Gb'tim*) in the optic lobe in DD, explaining the persistence of the locomotor rhythm. Surprisingly, *cyc* RNAi revealed a rhythmic expression of *Gb'Clk* in DD which is otherwise rather constitutively expressed in the optic lobe. These facts suggest that the cricket might have a unique clock oscillatory mechanism in which both *Gb'cyc* and *Gb'Clk* are rhythmically controlled and that under abundant expression of *Gb'cyc* the rhythmic expression of *Gb'Clk* may be concealed.

3.2. Introduction

Most insects live in harmony with the natural daily cycle to show a daily rhythm in their behavior. The rhythm is regulated by an endogenous mechanism called circadian clock which generates a 24 h oscillation. The oscillatory mechanism of the circadian clock is most extensively studied in the fruit fly *Drosophila melanogaster*, and thought to consist of interlocked autoregulatory transcriptional/ translational feedback loops involving a set of clock genes such as *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*) and *cycle* (*cyc*) (Hardin, 2009; Tomioka and Matsumoto, 2010). In brief, following transcription of *per* and *tim*, their product proteins PER and TIM accumulate in the cytoplasm during the night, form PER/TIM heterodimer to enter the nucleus, and inactivate heterodimeric transcription factors CLK/CYC to repress their own transcription (Sehgal et al., 1994; Lee et al., 1999). This negative feedback generates the rhythmic expression of *per* and *tim*. The *Clk* gene is also rhythmically expressed by a loop including *vrille* (*vri*) and *Par domain protein 1ε* (*Pdp1ε*) (Cyran et al., 2003; Glossop et al., 2003). CLK/CYC activates the transcription of both *vri* and *Pdp1ε* but *vri* mRNA peaks prior to the peak of *Pdp1ε* mRNA. The VRI protein thus accumulating earlier represses the *Clk* transcription, while PDP1ε later stimulates the transcription of *Clk* by competitive binding to VRI/PDP1ε box with VRI, making *Clk* to peak during the early day (Cyran et al., 2003; Glossop et al., 2003; Hardin, 2005). The third loop produces a rhythmic expression of *clockwork orange* (*cwo*) (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007). These three loops are thought to be coupled to produce a stable circadian oscillation.

However, immunohistochemical and molecular studies have yielded noticeably different results among insects, suggesting that insect clocks have considerably diversified. For example, there is no evidence that PER enters the nucleus in moths, bugs and cockroaches (Sauman and Reppert, 1996; Vafopoulou et al., 2007; Wen and Lee, 2008; Zavodska et al., 2008), and *tim* is absent in the genome of hymenopteran species (Rubin et al., 2006; Zhan et al., 2011). In addition, some insects show rhythmic expression of *cyc* instead of *Clk* (Rubin et al., 2006; Kamae et al., 2010). To understand how the variety of clock mechanisms derived from an ancestral one, further comparative study is necessary.

In the cricket *Gryllus bimaculatus*, *per* and *Clk* are essential components of the clock because their RNA interference (RNAi) stops the molecular oscillation as well as the locomotor rhythm (Moriyama et al., 2008, 2012). In contrast to *Drosophila*, however, *Clk* is rather constitutively expressed in the optic lobe, suggesting a cyclic expression of its partner, *cyc*.

In this study, cDNA of the clock gene *cycle* (*Gb'cyc*) was cloned from the cricket *G. bimaculatus*, examined its daily expression pattern using quantitative real-time RT-PCR (qPCR), and investigated its role in circadian oscillatory mechanism with RNAi. The results showed that the sequence of *Gb'cyc* showed a similarity to that of its mammalian orthologue, *Bmal1* and that its expression was rhythmic in light dark (LD) cycles. Interestingly, its knockingdown by RNAi resulted in a lengthening of free-running period of locomotor rhythms and revealed a rhythmic expression of *Gb'Clk*. These results suggest not only that *Gb'cyc* is an important component of the cricket's clock but also that the

cricket might possess a unique circadian clock including a mechanism for rhythmic expression of both *cyc* and *Clk* in certain conditions.

3.3. Materials and methods

Animals

Adult male crickets, *G. bimaculatus*, were used. They were obtained from a laboratory colony maintained under standard environmental conditions with a light dark (LD) cycle of 12 h light to 12 h dark (light: 06:00–18:00 h; Japanese standard time, JST) at a constant temperature of 25.0 ± 0.5 ^{o}C. They were fed laboratory chow and water.

Cloning and structural analysis of the clock gene *cyc*

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) from 6 adult male optic lobes collected at ZT 14 (ZT stands for zeitgeber time and ZT0 corresponds to lights-on and ZT12 to lights-off). 5 μg of total RNA was used for reverse transcription to obtain cDNA, using SuperScript II (Invitrogen, Carlsbad, CA). Using the single-stranded cDNA as a template, PCR was performed with degenerate primers deduced from the conserved amino acid sequences among insect *cyc* orthologues. The primers used were 5′-CAAGAGATGGCGAGATAAGATGAAYACNTA-3′ for forward, and 5′-TCATNCGRCARAARAA-3′ for reverse. The PCR conditions employed were: 30 s for denaturation at 95 ^{o}C, 30 s for annealing at 55 ^{o}C, and 90 s for extension at 72 ^{o}C for 35 cycles with ExTaq DNA polymerase (Takara, Ohtsu, Japan). The purified fragment was cloned into TOPO-pCR II vector (Invitrogen)

and sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). 5′ and 3′ RACEs were done with GeneRacer™ kit (Invitrogen) and SMARTer™ RACE cDNA Amplification kit (Takara) with gene specific primers, 5′-ACATGCTCCGTCAAAACAAGAG-3′ and 5′-GTCTGTATCGCAGGTGCTCA -3′, respectively. RACE fragments were purified, cloned and sequenced as mentioned above. Sequences were analyzed by Genetyx ver. 6 (Genetic Information Processing Software, Tokyo, Japan) and BioEdit ver. 7.0.5 (Biological Sequence Alignment Editor, Ibis Therapeutic, Carlsbad, CA). CYC's amino acid sequences were analyzed with ClustalW (http://clustalw.ddbj.nig.ac.jp/top-j.html). Sequences of known insect *cyc*s were obtained from GenBank.

Measurement of mRNA levels

Quantitative real-time RT-PCR (qPCR) was used to measure mRNA levels. Total RNA was extracted and purified from 6 adult male optic lobes with TRIzol® Reagent (Invitrogen). To remove contaminating genomic DNA, the total RNA was treated with DNase I. About 500 ng total RNA of each sample was reverse transcribed with random 6mers using PrimeScript™ RT reagent Kit (Takara). Real-time PCR was performed by Mx3000P™ Real-Time PCR System (Stratagene, La Jolla, CA) using FastStart Universal SYBR Green Master (Roche, Tokyo, Japan) including SYBER Green with primers 5′-GGCCGAAGCTCATAAAGTGG-3′ and 5′-AACCGCACAAAGGAACCATC-3′ for *Gb'cyc*, 5′-GATTATGAAGTCTGTGATGATTGG-3′ and 5′-AGCATTGGAGAGAACTGAAGAGGT-3′ for *Gb'tim*, and with those

described previously for *Gb'Clk*, *Gb'per*, and *Gb'rpl18a* (Moriyama et al., 2012). The results were analyzed using the software associated with the instrument. The values were normalized with the values of *Gb'rpl18a* at each time point. Results of three to eight independent experiments were used to calculate the mean ± SEM.

RNAi

Double stranded RNA (dsRNA) for *Gb'cyc*, *Gb'Clk*, *Gb'per* and *DsRed2*, derived from a coral species (*Discosoma* sp.), were synthesized using MEGAscript® High Yield Transcription Kit (Ambion, Austin, TX, USA). For *Gb'cyc*, *Gb'Clk* and *Gb'per*, using cDNA, prepared as described above as a template, PCR was performed with ExTaq DNA polymerase (Takara). The primers used were 5'-TAATACGACTCACTATAGGGCGTGCACTCGTACACTGAGG-3' for forward, and 5'-AATTAACCCTCACTAAAGGGAGGTTCTGCTGCTTCT TTCG-3' for reverse for *Gb'cyc* (ds*cyc* #1), 5'-TAATACGACTCACTATAGGGATGGAAATCCTTCCGAAACC-3' for forward, and 5'-AATTAACCCTCACTAAAGGGATCCCACCTCTTTGATGCAC-3' for reverse for another region of the *Gb'cyc* (ds*cyc* #2). The primers for *Gb'per*, *Gb'Clk* and *DsRed2* were those described in Moriyama et al., (2012). Amplified *Gb'cyc* (#1 450 bp and #2 529 bp) fragments were linearized and precipitated with ethanol. For *DsRed2* dsRNA, *DsRed2* was linearized from pDsRed2-N1 (Clontech, Mountain View, CA, USA), amplified with the forward and reverse primers. With each of these linearized fragments as a template, RNA was

synthesized and dsRNAs were prepared and stored at -80 °C as described previously (Moriyama et al., 2012). 760 nl of dsRNA solution was injected with the nanoliter injector (WPI, Sarasota, FL, USA) into the abdomen of adults anesthetized with CO_2. Otherwise noted, the injection was made within 3 days after the imaginal molt, and the optic lobes were collected seven days after the injection for the measurement of mRNA levels.

Behavioral analysis

Locomotor activities were recorded in the same way as described previously (Moriyama et al., 2008). Briefly, adult crickets were individually housed in a transparent plastic box (18 × 9 × 4.5 cm) with a rocking substratum. The number of substratum rocking was recorded every 6 min by a computerized system. Food and water were provided ad libitum. The locomotor activity was monitored in an incubator (MIR-153, Sanyo Biomedica, Osaka, Japan), in which temperature was kept at 25 °C and lighting conditions were given by a cool white fluorescent lamp connected to an electric timer. The light intensity was 600–1000 lux at the animal's level, varying with the proximity to the lamp. The raw data were displayed as conventional double-plotted actograms to judge activity patterns, and statistically analyzed by the chi-square periodogram (Sokolove and Bushell, 1978) with Actogram J (http://actogramj.neurofly.de/) (Schmid et al., 2011). If a peak of the periodogram appeared above the 0.05 confidence level (alpha = 0.005), the power value (height of the peak above the confidence level) was greater than or equal to 10, and the width of the peak was greater than or equal to 2, the period for the peak was designated as statistically significant (Kaneko et al., 2000).

3.4. Results

Cloning and structural analysis of *Gb'cyc*

A *cyc* was cloned homologue from the optic lobe of *G. bimaculatus* by degenerate PCR strategy with primers designed from conserved amino acid sequences of known insect's *cyc* orthologues. A 516 bp of *cyc* fragment including a part of bHLH and PAS-A domain was first obtained. Then 3′ and 5′ RACEs were performed with gene specific primers designed from the obtained sequence. The obtained full length cDNA was of 1967 bp encoding a protein of 599 aa residues with 100 and 67 bp of 3′- and 5′-UTR, respectively. A BLAST database search indicated that the amino acid sequence has 49–78% identities along the entire length of the protein with those of known insects' *cyc* orthologues (Table 3-1), including the firebrat *Thermobia domestica* (GenBank/EMBL/DDBJ accession No. BAJ16354), the honeybee *Apis mellifera* (XP_001121441), the beetle *Tribolium castaneum* (NP_001107795), the monarch butterfly *Danaus plexippus* (AAR13012) and the fruit fly *D. melanogaster* (NP_524168). The deduced protein had four highly conserved regions that are characteristic of known CYC proteins (Fig. 3-1A and B): (i) bHLH domain, (ii) PAS-A domain, (iii) PAS-B domain, (iv) BMAL1 C-terminal region (BCTR) in C-termini. The BCTR, which shows potent transcriptional activity *in vitro* (Takahata et al., 2000; Chang et al., 2003; Chang and Reppert, 2003), has a particularly high similarity (83–90% identity) to those of known insect CYC (Table 3-1). Based on these identities and similarities, this gene was concluded to encode a cricket's CYC (*Gb'cyc*; Gen- Bank/EMBL/DDBJ accession No.

AB762416). A phylogenetic tree based on the amino acid sequences of CYCs from known insect species revealed that *Gb'cyc* forms a separate bifurcation (Fig. 3-1C).

Gb'cyc expression in the optic lobe under LD and DD

To determine if *Gb'cyc* transcript oscillated in a circadian manner, the levels of *Gb'cyc* mRNA in the optic lobe were examined under LD12:12 and in the second day after transfer to constant darkness (DD) by qPCR (Fig. 3-2). The *Gb'cyc* mRNA level showed a significant fluctuation peaking at ZT18 in LD (ANOVA, $P < 0.05$). A slight increase was observed during the midday but was not significant (Tukey-test, $P > 0.05$). In DD, however, there was no significant daily fluctuation (ANOVA, $P > 0.05$; Fig. 3-2B).

Gb'cyc dsRNA suppresses levels of *Gb'cyc* transcripts

To examine the effects of *Gb'cyc* dsRNA on *Gb'cyc* expression, the *Gb'cyc* mRNA levels were measured by qPCR in the optic lobe of the adult male crickets injected with ds*cyc*#1 (Fig. 3-2). In the control crickets treated with *DsRed2* dsRNA (ds*DsRed2*), the *Gb'cyc* mRNA levels were slightly reduced but no significant difference was observed when compared with those of intact crickets in LD (Fig. 3-2A). *Gb'cyc* RNAi significantly reduced *Gb'cyc* mRNA levels in LD to nearly 25% of the peak level of intact crickets throughout the day (Fig. 3-2A, *t*-test, $P < 0.05$). Similar significant reduction was observed in DD (*t*-test, $P < 0.05$): the *Gb'cyc* mRNA levels stayed below the trough level of intact crickets throughout the day (Fig. 3-2B). Although slight fluctuations in *Gb'cyc* mRNA levels were observed in both LD and DD, these were statistically not

significant (ANOVA, $P > 0.1$). To exclude off-target effects of dsRNA, the *Gb'cyc* mRNA levels were measured after injection with ds*cyc*#2 synthesized from a different region of the *Gb'cyc* cDNA. The results were similar to those treated with ds*cyc*#1: *cyc* mRNA levels were significantly reduced (Fig. 3-2A, *t*-test, $P < 0.01$). It is thus evident that ds*cyc* successfully suppressed the expression of *Gb'cyc* mRNA through RNAi.

Gb'cyc RNAi lengthened free-running period of the locomotor rhythm

To examine the role of *Gb'cyc* in the circadian rhythm generation, the effects of ds*cyc* on locomotor rhythms were examined in male crickets. Effects of ds*cyc*#1 and ds*cyc*#2 were examined in 55 and 20 adult crickets, respectively. As a control *DsRed2* dsRNA was injected in the abdomen of 17 adult crickets. Locomotor rhythms were also measured in 12 intact males. Both the ds*cyc* injected and the ds*DsRed2* injected control crickets exhibited an activity rhythm under LD12:12, with peaks at lights-on and sometimes in mid light phase in the early adult stage, thereafter an intense activity peak appeared at lights-off (Fig. 3-3A, B and C). Similar locomotor rhythms were observed in intact crickets (data not shown), as previously reported (Tomioka and Chiba, 1982). On transfer to DD, all the crickets showed a free-running rhythm persisting from the light-off component (Fig. 3-3). The average free-running periods of ds*cyc* injected crickets were 24.9 ± 0.4 h for both ds*cyc*#1 (n = 31) and ds*cyc*#2 (n = 14), which were significantly longer than that of intact (23.6 ± 0.2, n = 12) and control crickets injected with *DsRed2* dsRNA (23.8 ± 0.4 h, n = 14) (*t*-test, $P < 0.01$, Table 3-2). There was no marked difference in power of the rhythm between control and *Gb'cyc* RNAi crickets both under LD and DD (Table3- 2).

Effects of *Gb'cyc* dsRNA on *Gb'per*, *Gb'tim* and *Gb'Clk* transcripts

In *Drosophila*, CYC is known to form a heterodimer with CLK to activate the transcription of *per* and *tim* (Rutila et al., 1998). To examine whether *Gb'cyc* is involved in the regulation of these genes, the levels of *Gb'per*, *Gb'tim* and *Gb'Clk* mRNA in the optic lobe were measured by qPCR in adult male crickets seven days after injection of ds*cyc*#1. In intact crickets, the *Gb'per* and *Gb'tim* showed a clear rhythm peaking in the mid night phase in LD as have been reported previously (Moriyama et al., 2008; Danbara et al., 2010) and their mRNA levels were significantly reduced by *Gb'cyc* RNAi at ZT2, 10–18 (*t*-test, $P < 0.01$) for *Gb'per* and at ZT14 (*t*-test, $P < 0.01$) for *Gb'tim* (Fig. 3-4A and B). No clear rhythms were observed in both *per* and *tim* mRNA. The *Gb'Clk* mRNA showed no significant rhythm in intact crickets kept in LD as reported by Moriyama et al., (2012) and the ds*cyc* treatment significantly reduced *Gb'Clk* mRNA levels at most time points (ZT6, 14, 18; *t*-test, $P < 0.05$, Fig. 3-4C). In constant darkness (DD), however, these clock genes showed a rhythmic expression, even after the ds*cyc* treatment. The *Gb'per* and *Gb'tim* peaked at CT18 in intact crickets and similar rhythms were observed in *cyc* RNAi crickets for both *Gb'per* and *Gb'tim* (Fig. 3-4D and E, ANOVA, $P < 0.05$). These molecular rhythms are consistent with the free-running locomotor rhythm in DD. Surprisingly, the *Gb'Clk* also showed a significantly rhythmic expression with a peak at CT14 (Fig. 3-4F, ANOVA, $P < 0.05$), although it showed no significant rhythm in intact crickets (Fig. 3-4F, Moriyama et al., 2012).

Effects of ds*per* and ds*Clk* on *Gb'cyc* mRNA levels

The effects of ds*per* and ds*Clk* on expression of *Gb'cyc* were then examined. The knock-down of respective mRNA by ds*per* and ds*Clk* through RNAi has been confirmed previously (Moriyama et al., 2008, 2012). When *Gb'per* was knocked-down, the *Gb'cyc* mRNA level was significantly downregulated (*t*-test, $P < 0.05$) except for ZT22 and stayed below the basal level of intact animals throughout the day, showing no statistically significant rhythm (ANOVA, $P > 0.05$, Fig. 3-5). In *Clk* RNAi, *Gb'cyc* mRNA levels were again significantly reduced at ZT 6, 10 and 18 (*t*-test, $P < 0.05$) and showed no statistically significant rhythm (ANOVA, $P > 0.05$, Fig. 3-5).

3.5. Discussion

In the present study, the structure, expression profile, and function of the clock gene *cyc* were investigated in a hemimetabolous insect, *G. bimaculatus*. The deduced *Gb'*CYC protein has 4 functional domains, i.e. bHLH, PAS-A, PAS-B and BCTR domains (Fig. 3-1), which are commonly observed among known insect CYC orthologues (Bembenek et al., 2007). The BCTR domain, which is missing in *Drosophila* CYC, has particularly high homology with CYC orthologues from other insects, supporting the view that the BCTR is evolutionarily conserved in insects, but it might have been somehow lost in *Drosophila* (Chang et al., 2003).

The BCTR domain has been shown to have potent transcriptional activity *in vitro* (Takahata et al., 2000; Chang et al., 2003; Chang and Reppert,

2003). The result that *Gb'cyc* RNAi suppressed the mRNA levels of *Gb'per* (Fig. 3-4) is consistent with this hypothesis and also in good agreement with what is expected from the *Drosophila* clock model. It is most probable that *Gb'cyc* acts as a positive element in the clock machinery and *Gb'cyc* may directly transactivate *Gb'per*. Interestingly, however, in the *Gb'cyc* RNAi crickets the mRNA level of *Gb'tim* was knocked down in LD but maintained a clear rhythm in DD (Fig. 3-4). A similar rhythm was also seen in *Gb'per* in DD (Fig. 3-4). These facts suggest that there is a mechanism maintaining a rhythmic transcription of *Gb'per* and *Gb'tim* even after *Gb'cyc* is substantially knocked down and that the rhythmic expression of *Gb'per* and *Gb'tim* may be responsible for the locomotor rhythm with a lengthened free-running period (Fig. 3-3). *Gb'Clk* might be a candidate element responsible for this rhythmic transcription of *Gb'per* and *Gb'tim* because *Gb'Clk* was rhythmically expressed in the *Gb'cyc* RNAi crickets (Fig. 3-4) and *Gb'Clk* has poly-Q region which is likely involved in transcriptional activation (Allada et al., 1998; Moriyama et al., 2012). Another explanation for the free-running locomotor rhythms in DD might be that there is enough amount of residual *Gb'cyc* mRNA surviving the RNAi treatment for generation of the rhythm. An important issue remains to be answered is why clear rhythms of *Gb'per* and *Gb'tim* in the *cyc* RNAi treated crickets were observed only in DD (Fig. 3-4).

The *Gb'cyc* was rhythmically expressed in the optic lobe in LD (Fig. 3-2) and a similar and clear oscillation has been observed in the adult brain (Chapter 5). The rhythm in the optic lobe was abolished in *Gb'per* RNAi and *Gb'Clk* RNAi crickets (Fig. 3-5). These facts suggest that the rhythmic expression of *Gb'cyc* is

not a simple response to LD but regulated by a mechanism involving *Gb'per* and *Gb'Clk*. This hypothesis is conceivable given that rhythmic expression of *cyc* is also known for the firebrat (*T. domestica*), one of the most primitive insects, and for those insects phylogenetically higher than crickets such as the honeybee (*A. mellifera*) and the sandfly (*Lutzomyia longipalpis*) (Meireles-Filho et al., 2006a; Rubin et al., 2006; Kamae et al., 2010). Since the rhythmic expression of *Gb'cyc* disappeared in DD (Fig. 3-2B), the rhythm seems highly dependent on light cycles, however. It may be also possible that *Gb'cyc* is rhythmically expressed only in a subset of neurons that controls behavior and the rhythm is masked by constitutive expression in other non-pacemaker cells in DD. Further critical study is necessary to clarify the reason why the rhythmic expression became obscure in DD.

Interestingly, the *Gb'cyc* mRNA peaked in the mid night similar to those of *Gb'per* and *Gb'tim* (Figs. 3-2A, 3-4A and B). Coherence of the peak phase was also found in *Gb'Clk* mRNA in *cyc* RNAi crickets (Fig. 3-4F). A similar situation is observed in the sandfly *L. longipalpis*, in which *Clk* and *cyc* transcripts peak simultaneously with or slightly earlier than *per* and *tim* (Meireles-Filho et al., 2006a, 2009b) and also in some mammalian peripheral tissues where *Bmal1* and *per* are expressed almost in phase (Tong et al., 2004; Bebas et al., 2009). This feature is opposed to the *Drosophila* clock hypothesis, where peaks of *per*/*tim* and *Clk* transcription are about 12 h apart from each other (Hardin, 2009). The reason and mechanism underlying this phase coherence should be addressed in future studies.

The present study revealed that the cricket clock possesses a unique

feature in that *Gb'cyc* shows a clear rhythmic expression in LD and that *Gb'Clk* is expressed in a rhythmic fashion only when *Gb'cyc* is knocked-down (Figs. 3-2 and 3-4). In both vertebrates and insects, either *cyc* (*Bmal1*) or *Clk* is reportedly expressed in a rhythmic manner (Honma et al., 1998; Stanewsky, 2002; Hardin, 2009). In insects, *Clk* is known to oscillate in *Drosophila* while *cyc* does in other species (Cyran et al., 2003; Glossop et al., 2003; Meireles- Filho et al., 2006a; Rubin et al., 2006; Kamae et al., 2010). Although further experiments are necessary to confirm the rhythmic expression of *Gb'Clk* in *cyc* RNAi crickets, it should be worth examining whether the two mechanisms for rhythmic expression of *cyc* and *Clk* might coexist in the cricket. Since a more primitive insect, the firebrat, exhibits a rhythm in *cyc* but not in *Clk* expression (Kamae et al., 2010), it might provide a view on the evolutionary history of the insect circadian clock.

The data indicate that *Gb'per* is required for the sufficient expression of *Gb'cyc* at least in LD (Fig. 3-5). Because it is also required for *Gb'Clk* (Moriyama et al., 2012), *Gb'per* seems to play a common role in regulation of these two genes. One may explain that a reduced level of PER by *per* RNAi may increase the transcriptional activity of CLK/CYC since the CLK/CYC transcriptional activity is suppressed by PER/TIM (Stanewsky, 2002; Tomioka and Matsumoto, 2010). The increased activity of CLK/CYC would result in an increased level of transcriptional repressor like *vri* for *Clk*, which may eventually reduce the *cyc* and *Clk* transcript levels. While the transcriptional regulations of *Gb'Clk* and *Gb'cyc* in crickets should be elucidated in future, the results strongly suggest that both regulations are interlocked in the circadian feedback loop. It is

interesting to know the mechanism by which the two positive elements are regulated in transcription. Future studies would reveal a unique molecular feedback system that may be similar to neither *Drosophila* nor mammalian system.

References

Allada, R., White, N.E., So, W.V., Hall, J.C., Rosbash, M. (1998) A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*. Cell 93, 791–804.

Bebas, P., Goodall, C.P., Majewska, M., Neumann, A., Giebultowicz, J.M., Chappell, P.E. (2009) Circadian clock and output genes are rhythmically expressed in extratesticular ducts and accessory organs of mice. The FASEB Journal 23, 523– 533.

Bembenek, J., Itokawa, K., Hiragaki, S., Qi-Miao, S., Tufail, M., Takeda, M. (2007) Molecular characterization and distribution of CYCLE protein from Athalia rosae. Journal of Insect Physiology 53, 418–427.

Chang, D.C., McWatters, H.G., Williams, J.A., Gotter, A.L., Levine, J.D., Reppert, S.M. (2003) Constructing a feedback loop with circadian clock molecules from the silkmoth, *Antheraea pernyi*. Journal of Biological Chemistry 278, 38149–38158.

Chang, D.C., Reppert, S.M. (2003) A novel C-terminal domain of *Drosophila* PERIOD inhibits dCLOCK:CYCLE-mediated transcription. Current Biology 13, 758–762.

Cyran, S.A., Buchsbaum, A.M., Reddy, K.L., Lin, M.-C., Glossop, N.R.J., Hardin, P.E., Young, M.W., Storti, R.V., Blau, J. (2003) Vrille, Pdp1 and dClock form a second feedback loop in the *Drosophila* circadian clock. Cell 112, 329–341.

Danbara, Y., Sakamoto, T., Uryu, O., Tomioka, K. (2010) RNA interference of timeless gene does not disrupt circadian locomotor rhythms in the cricket *Gryllus bimaculatus*. Journal of Insect Physiology 56, 1738–1745.

Glossop, N.R., Houl, J.H., Zheng, H., Ng, F.S., Dudek, S.M., Hardin, P.E. (2003) VRILLE feeds back to control circadian transcription of clock in the *Drosophila* circadian oscillator. Neuron 37, 249–261.

Hardin, P. (2005) The circadian timekeeping system of *Drosophila*. Current Biology 15, R714–R722.

Hardin, P. (2009) Molecular mechanisms of circadian timekeeping in *Drosophila*. Sleep and Biological Rhythms 7, 235–242.

Honma, S., Ikeda, M., Abe, H., Tanahashi, Y., Namihira, M., Honma, K., Nomura, M. (1998) Circadian oscillation of BMAL1, a partner of a mammalian clock gene clock, in rat suprachiasmatic nucleus. Biochemical Biophysical Research Communications 250, 83–87.

Kadener, S., Stoleru, D., McDonald, M., Nawathean, P., Rosbash, M. (2007) Clockwork orange is a transcriptional repressor and a new *Drosophila* circadian pacemaker component. Genes and Development 21, 1675–1686.

Kamae, Y., Tanaka, F., Tomioka, K. (2010) Molecular cloning and functional analysis of the clock genes, clock and cycle, in the firebrat *Thermobia domestica*. Journal of Insect Physiology 56, 1291–1299.

Kaneko, M., Park, J.H., Cheng, Y., Hardin, P.E., Hall, J.C. (2000) Disruption of synaptic transmission or clock-gene-product oscillations in circadian pacemaker cells of *Drosophila* cause abnormal behavioral rhythms. Journal of Neurobiology 43, 207–233.

Lee, C., Bae, K., Edery, I. (1999) PER and TIM inhibit the DNA binding activity of a *Drosophila* CLOCK-CYC/dBMAL1 Heterodimer without disrupting formation of the Heterodimer: a Basis for circadian transcription. Molecular and Cellular Biology 19, 5316–5325.

Lim, C., Chung, B.Y., Pitman, J.L., McGill, J.J., Pradhan, S., Lee, J., Keegan, K.P., Choe, J., Allada, R. (2007) Clockwork orange encodes a transcriptional repressor important for circadian-clock amplitude in *Drosophila*. Current Biology 17, 1082–1089.

Matsumoto, A., Ukai-Tadenuma, M., Yamada, R.G., Houl, J., Uno, K.D., Kasukawa, T., Dauwalder, B., Itoh, T.Q., Takahashi, K., Ueda, R., Hardin, P.E., Tanimura, T., Ueda, H.R. (2007) A functional genomics strategy reveals clockwork orange as a transcriptional regulator in the *Drosophila* circadian clock. Genes and Development 21, 1687–1700.

Meireles-Filho, A.C.A., Amoretty, P.R., Souza, N.A., Kyriacou, C.P., Peixoto, A.A. (2006a) Rhythmic expression of the cycle gene in a hematophagous insect vector. BMC Molecular Biology 7, 38.

Meireles-Filho, A.C.A., Rivas, G.B.S., Gesto, J.S.M., Machado, R.C., Britto, C., de

Souza, N.A., Peixoto, A.A. (2006b) The biological clock of an hematophagous insect: locomotor activity rhythms, circadian expression and downregulation after a blood meal. FEBS Letters 580, 2–8.

Moriyama, Y., Kamae, Y., Uryu, O., Tomioka, K. (2012) *Gb'Clock* is expressed in the optic lobe and required for the circadian clock in the cricket *Gryllus bimaculatus*. Journal of Biological Rhythms 27, 467–477.

Moriyama, Y., Sakamoto, T., Karpova, S.G., Matsumoto, A., Noji, S., Tomioka, K. (2008) RNA interference of the clock gene period disrupts circadian rhythms in the cricket *Gryllus bimaculatus*. Journal of Biological Rhythms 23, 308–318.

Rubin, E.B., Shemesh, Y., Cohen, M., Elgavish, S., Robertson, H.M., Bloch, G. (2006) Molecular and phylogenetic analyses reveal mammalian-like clockwork in the honey bee (*Apis mellifera*) and shed new light on the molecular evolution of the circadian clock. Genome Research 16, 1352–1365.

Rutila, J.E., Suri, V., Le, M., So, W.V., Rosbash, M., Hall, J.C. (1998) CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. Cell 93, 805–814.

Sauman, I., Reppert, S.M. (1996) Circadian clock neurons in the silkmoth *Antheraea pernyi*: novel mechanisms of period protein regulation. Neuron 17, 889–900.

Schmid, B., Helfrich-Forster, C., Yoshii, T. (2011) A new ImageJ plug-in ''ActogramJ'' for chronobiological analyses. Journal of Biological Rhythms 26, 464–467.

Sehgal, A., Price, J.L., Man, B., Young, M.W. (1994) Loss of circadian behavioral rhythms and *per* RNA oscillations in the *Drosophila* mutant timeless. Science 263, 1603–1606.

Sokolove, P.G., Bushell, W.N. (1978) The chi square periodogram: its utility for analysis of circadian rhythm. Journal of Theoretical Biology 72, 131–160.

Stanewsky, R. (2002) Clock mechanisms in *Drosophila*. Cell and Tissue Research 309, 11–26.

Takahata, S., Ozaki, T., Mimura, J., Kikuchi, Y., Sogawa, K., Fujii-Kuriyama, Y. (2000) Transactivation mechanisms of mouse clock transcription factors, *mClock* and *mArnt3*. Genes to Cells 5, 739–747.

Tomioka, K., Chiba, Y. (1982) Post-embryonic development of circadian rhythm in the cricket, *Gryllus bimaculatus*. Journal of Comparative Physiology A 147, 299– 304.

Tomioka, K., Matsumoto, A. (2010) A comparative view of insect circadian clocks. Cellular and Molecular Life Sciences 67, 1397–1406.

Tong, Y., Guo, H., Brewer, J.M., Lee, H., Lehman, M.N., Bittman, E.L. (2004) Expression of *haPer1* and *haBmal1* in Syrian hamsters: heterogeneity of transcripts and oscillations in the periphery. Journal of Biological Rhythms 19, 113–125.

Vafopoulou, X., Steel, C.G.H., Terry, K.L. (2007) Neuroanatomical relations of prothoracicotropic hormone neurons with the circadian timekeeping system in the brain of larval anxd adult *Rhodnius prolixus* (Hemiptera). Journal of Comparative Neurology 503, 511–524.

Wen, C.-J., Lee, H.-J. (2008) Mapping the cellular network of the circadian clock in two cockroach species. Archives of Insect Biochemistry and Physiology 68, 215– 231.

Zavodska, R., Wen, C.-J., Hrdy, I., Sauman, I., Lee, H.-J., Sehnal, F. (2008) Distribution of corazonin and pigment-dispersing factor in the cephalic ganglia of termites. Arthropod Structure Development 37, 273–286.

Zhan, S., Merlin, C., Boore, J.L., Reppert, S.M. (2011) The monarch butterfly genome yields insights into long-distance migration. Cell 147, 1171–1185.

Table 3-1. Overall amino acid identity (%) of whole sequence and functional domains of *Gryllus bimaculatus* CYCLE with those insect orthologues.

Species	Whole sequence	bHLH	PAS-A	PAS-B	BCTR
Thermobia domestica	78	74	94	87	90
Tribolium castaneum	63	72	89	79	88
Danaus plexippus	49	63	70	64	83
Drosophila melanogaster	62	61	81	69	—
Apis mellifera	78	74	92	89	90

Table 3-2. Effects of ds*cyc* and ds*DsRed2* on the locomotor rhythm of the cricket *Gryllus bimaculatus*.

Treatment	n	Period/Free-running period (mean±SD) h	Power (mean±SEM)
LD			
intact	12	24.0±0.14	485.1±19.4
DsRed2 dsRNA	17	24.0±0.07	150.6±19.4
cyc dsRNA(#1)	55	24.0±0.1	207.7±18.2
cyc dsRNA(#2)	20	24.0±0.06	181.4±25.9
DD			
intact	12	23.6±0.2	342.0±40.0
DsRed2 dsRNA	14	23.8±0.4	380.9±84.1
cyc dsRNA(#1)	31	24.9±0.4**	290.8±32.8
cyc dsRNA(#2)	14	24.9±0.4**	304.9±46.5

** $P < 0.01$, *t*-test vs *DsRed2* dsRNA treatment.

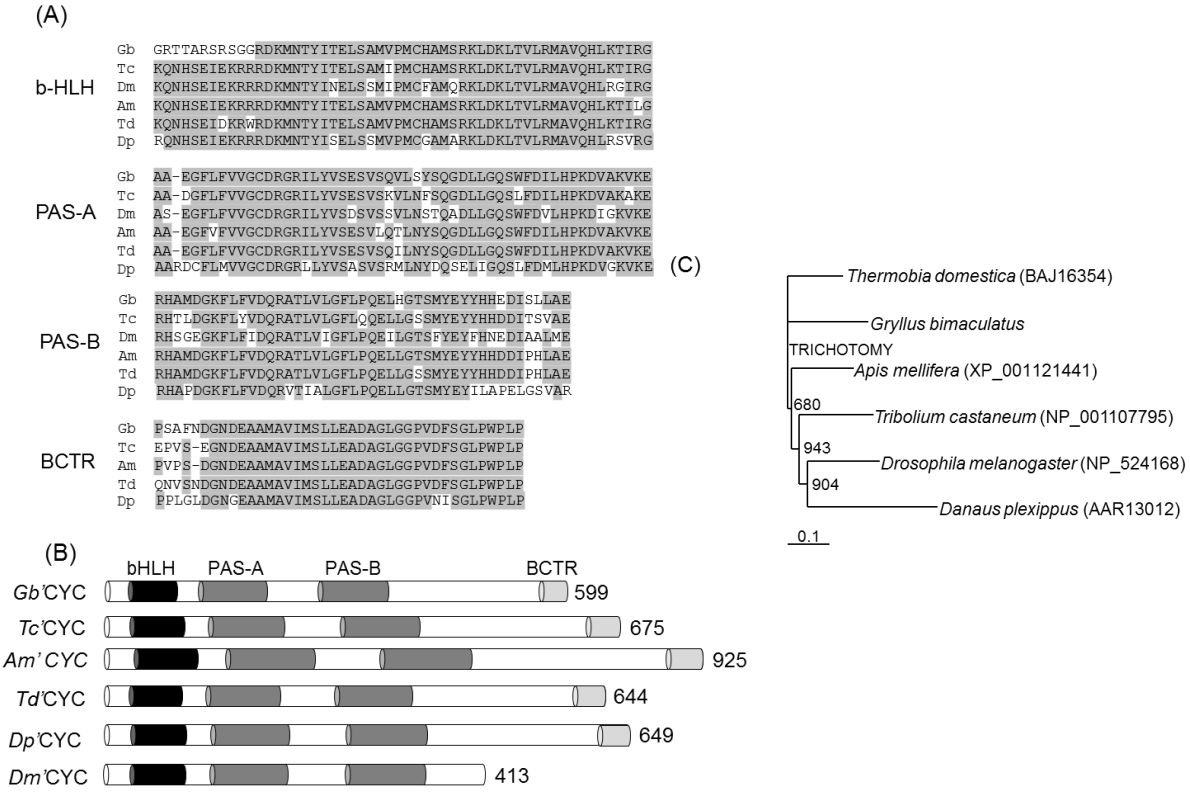

Fig. 3-1. Sequence alignments of conserved domains of *Gb'cyc* compared with those orthologous sequences from other insects.

(A) bHLH, PAS-A, PAS-B and BCTR of *Gryllus bimaculatus cyc* (*Gb*) aligned with those of *Tribolium castaneum* (*Tc*), *Drosophila melanogaster* (*Dm*) that lacks BCTR domain, *Apis mellifera* (*Am*), *Thermobia domestica* (*Td*) and *Danaus plexippus* (*Dp*). The amino acid residues showing more than 60% identity are shaded. (B) Schematic structure of various CYC proteins, comparing the organization of the 4 conserved domains, bHLH, PAS-A, PAS-B, and BCTR. The numbers on the right indicate number of amino acid residues. (C) A phylogenetic neighbor-joining tree of known insect CYC proteins. CYC amino acid sequences were analyzed and neighbor-joining tree was inferred with ClustalW. The GenBank or RefSeq accession numbers are indicated in the bracket. A reference bar indicates distance as number of amino acid substitutions per site. The neighbor-joining tree has a basal trichotomy (i.e., a three-way split at the root).

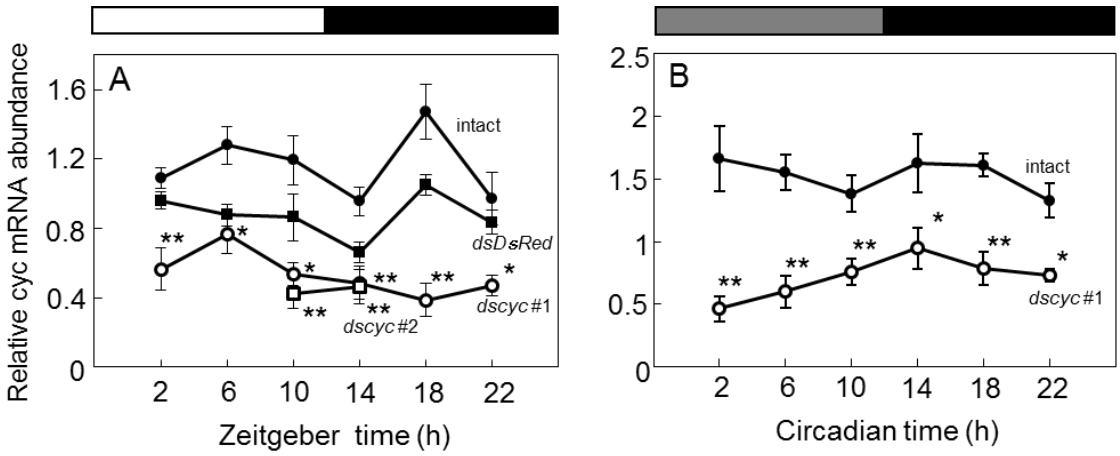

Fig. 3-2. Expression profiles of *Gb'cyc* mRNA in the *Gryllus bimaculatus* optic lobes and effects of *Gb'cyc* dsRNA under LD 12:12 (A) and DD (B). Filled circles, filled squares, open circles and squares indicate the mRNA levels of intact, *DsRed2* dsRNA, *cyc#1* dsRNA (ds*cyc#1*) and ds*cyc#2* injected crickets, respectively. The abundance of *Gb'cyc* mRNA was measured by quantitative real-time RT-PCR. Total RNA was extracted from the optic lobes that were collected at 4 h interval starting at 2 h after light-on (Zeitgeber time 2) or 2 h after projected light-on (Circadian time 2) in the second day of DD. White, black and gray bars indicate light phase, night/subjective night and subjective day, respectively. The data collected from 3~8 independent experiments were averaged and plotted as mean ± SEM. The abundance of *Gb'rpl18a* mRNA was used as an internal reference. In intact crickets, *Gb'cyc* mRNA levels showed significant daily changes (ANOVA, $P < 0.05$) to peak at ZT18 (A), while no significant rhythm was detected in DD (B) (ANOVA, $P>0.05$). Note that both ds*cyc#1* and ds*cyc#2* significantly reduced *cyc* mRNA levels and abolished the rhythm both in LD and DD (ANOVA, $P > 0.05$). *$P < 0.05$, **$P < 0.01$, *t*-test, vs intact crickets.

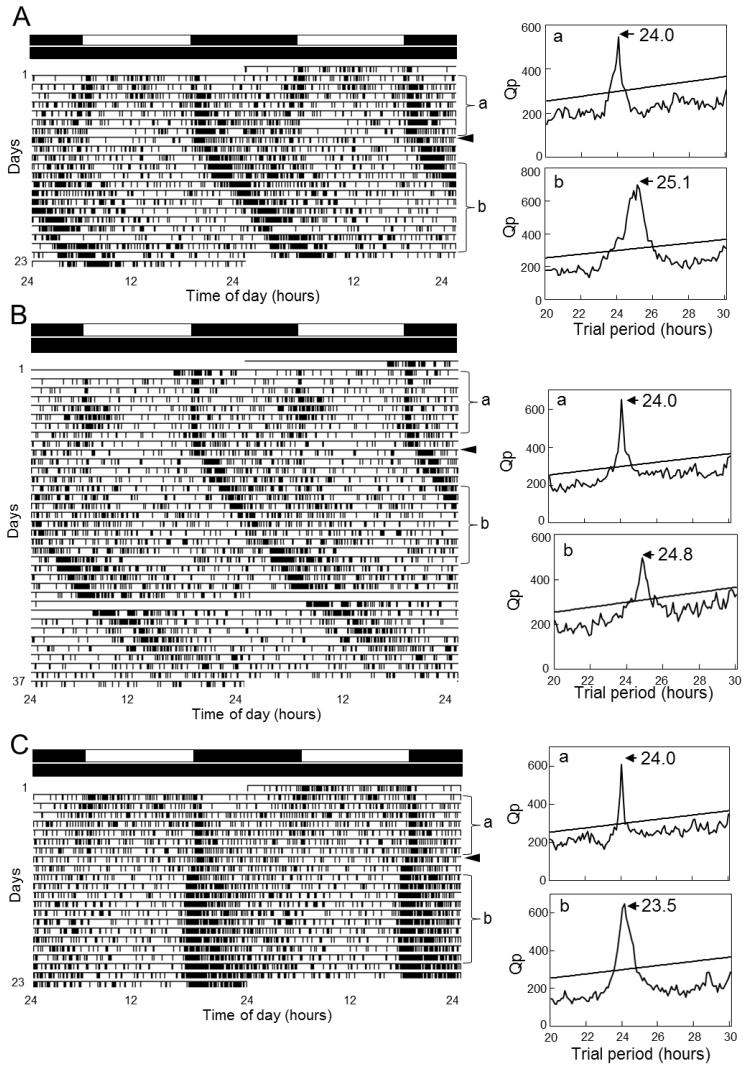

Fig. 3-3. Double-plotted actograms (left) and χ^2 periodograms (right) of locomotor rhythms of crickets *Gryllus bimaculatus* injected with *Gb'cyc* dsRNA [ds*cyc*#1 (A) or ds*cyc*#2 (B)], or with *DsRed2* dsRNA (C) under LD12:12 and DD at a constant temperature of 25 ºC.

Arrowheads indicate the day when the crickets were transferred from LD to DD. a and b indicated in the periodogram correspond to the analyzed time span, a and b, indicated in the actogram. An oblique line in the periodogram indicates significance level of $P < 0.05$; peak value above the line was designated as significant. The *cyc* RNAi crickets showed locomotor rhythm free-running with a period longer than 24 h in DD (A, B), but the control cricket injected with *DsRed2* dsRNA showed a rhythm with a period shorter than 24 h (C). For further explanations see text.

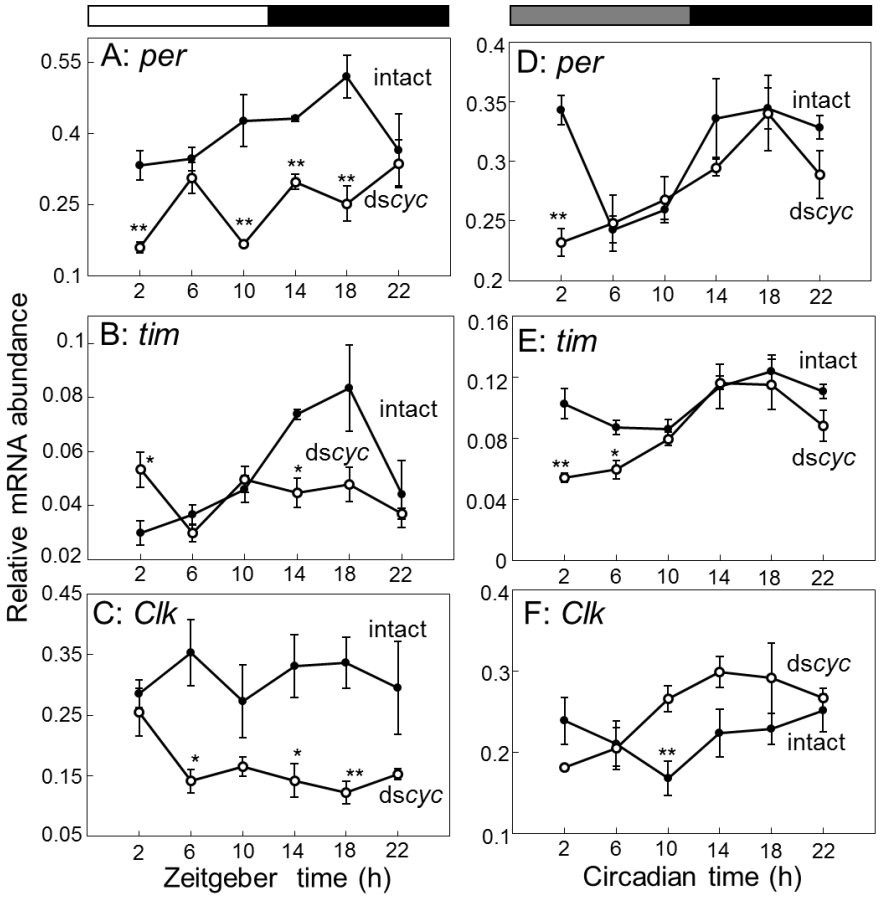

Fig. 3-4. Relative abundance of *Gb'per* (A, D), *Gb'tim* (B, E) or *Gb'Clk* mRNA (C, F) in the optic lobes of intact and of *cyc* double-stranded RNA (dsRNA)-injected adult male crickets *Gryllus bimaculatus* under LD (A-C) or DD (D-F).

Filled and open circles indicate the mRNA levels of intact and *Gb'cyc* dsRNA injected crickets, respectively. In *Gb'cyc* RNAi crickets, the optic lobes were collected 7 days after the dsRNA injection. The abundance of mRNA was measured by qPCR with total RNA extracted from the optic lobes. The data collected from 3~8 independent experiments were averaged and plotted as mean ± SEM for dsRNA-injected and intact crickets, respectively. The abundance of *Gb'rpl18a* mRNA was used as an internal reference. **P* < 0.05, ***P* < 0.01, *t*-test vs intact crickets. Note that *Gb'cyc* dsRNA down-regulated *Gb'per, Gb'tim* and *Gb'Clk* mRNA and prevents the rhythmic expression of *Gb'per* and *Gb'tim* while revealed a rhythmic expression in *Gb'Clk* in LD (ANOVA, *P* < 0.05). In DD, however, *Gb'per, Gb'tim* and *Gb'Clk* mRNAs oscillate clearly (ANOVA, *P* < 0.05 for *Gb'per* and *Gb'Clk*, *P* < 0.01 for *Gb'tim*). For further explanations see text.

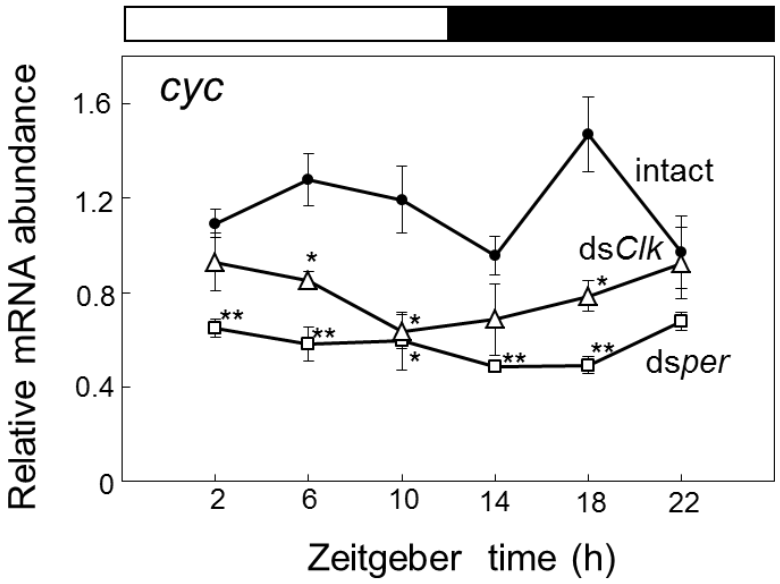

Fig. 3-5. Relative abundance of *cyc* mRNA in the optic lobes of intact crickets and those injected with dsRNA of *per* or *Clk* in LD. Filled circle, open square and triangle indicate the mRNA levels of intact, ds*per*-injected and ds*Clk*-injected crickets, respectively.

The data for intact crickets are replotted from Fig. 2A. **P* < 0.05, ***P* < 0.01, *t*-test vs intact crickets. Note that *Gb'per* and *Gb'Clk* dsRNAs down-regulate the *Gb'cyc* mRNA levels and abolished the daily expression rhythm (ANOVA, *P* > 0.05). For further explanations see text.

Chapter 4.

Circadian oscillations outside the optic lobe in the cricket *Gryllus bimaculatus*

4.1. Abstract

Although circadian rhythms are found in many peripheral tissues in insects, the control mechanism is still to be elucidated. To investigate the central and peripheral relationships in the circadian organization, circadian rhythms outside the optic lobes were examined in the cricket *Gryllus bimaculatus* by measuring mRNA levels of *period* (*Gb'per*) and *timeless* (*Gb'tim*) genes in the brain, terminal abdominal ganglion (TAG), anterior stomach, mid-gut, testis, and MTs. Except for MTs and testis, the tissues showed a daily rhythmic expression in either both *Gb'per* and *Gb'tim* or *Gb'tim* alone in LD. Under constant darkness, however, the tested tissues exhibited rhythmic expression of *Gb'per* and *Gb'tim* mRNAs, suggesting that they include a circadian oscillator. The amplitude and the levels of the mRNA rhythms varied among those rhythmic tissues. Removal of the optic lobe, the central clock tissue, differentially affected the rhythms: the anterior stomach lost the rhythm of both *Gb'per* and *Gb'tim*; in the mid-gut and TAG, *Gb'tim* expression became arrhythmic but *Gb'per* maintained rhythmic expression; a persistent rhythm with a shifted phase was observed for both *Gb'per* and *Gb'tim* mRNA rhythms in the brain. These data suggest that rhythms outside the optic lobe receive control from the optic lobe to different degrees, and that the oscillatory mechanism may be different from that of *Drosophila*.

4.2. Introduction

Insects show rhythmic lives to adapt daily cyclic environment

(Saunders, 2002). Those daily rhythms are controlled by an endogenous mechanism called the circadian clock. The locus of the clock has been identified in some insects. For example, it is the optic lobe in crickets (Tomioka and Chiba, 1992), cockroaches (Page, 1982), and beetles (Balkenohl and Weber, 1981), but it is within the central brain in flies (Handler and Konopka, 1979), mosquitoes (Kasai and Chiba, 1987) and moths (Truman, 1974). In addition to these central clock tissues regulating activity rhythms, peripheral tissues also show some rhythms. The compound eyes have a circadian rhythm in their responsiveness to light in crickets (Tomioka and Chiba, 1982a) and cockroaches (Wills et al., 1985), and sperm release from testis to the vas deferens in moths occurs in a circadian rhythmic manner (Giebultowicz et al., 1989). Antennal odor sensitivity is also under regulation of the circadian clock in cockroaches and flies (Krishnan et al., 2001; Page and Koelling, 2003). Cuticle secretion also occurs in a rhythmic manner in cockroaches (Wiedenmann et al., 1986) and flies (Ito et al., 2008).

The relationship between the central and peripheral clocks has been investigated in only limited species, however. In *Drosophila*, the peripheral oscillators are almost independent from the central clock, and they show a persistent rhythm even after being isolated and cultured *in vitro* (Plautz et al., 1997). *Drosophila* MTs transplanted to a host, which has a rhythm with an antiphase relationship to the donor, maintained its own phase for a long period (Giebultowicz et al., 2000). On the other hand, the cockroach antennal response to odors and compound eye sensitivity to light are apparently controlled by the central clock (Wills et al., 1985; Page and Koelling, 2003). However, the

mechanisms through which the peripheral rhythm is regulated by the central clock are still largely unknown.

In this study, circadian rhythms were investigated in tissues beyond the optic lobe in the cricket using mRNA expression of the clock genes, *period* (*Gryllus bimaculatus period*, *Gb'per*) and *timeless* (*G. bimaculatus timeless*, *Gb'tim*), which are major players in the oscillatory mechanism of *Drosophila* (cf. Stanewsky, 2002; Hardin, 2006). The relationship between rhythms outside the optic lobe and the central clock in the optic lobe was also examined. It was found that some tissues show rhythmic expression of the clock genes both under a light–dark cycle and under constant darkness. After optic lobe removal, the brain showed a persistent rhythm of both *Gb'per* and *Gb'tim* mRNAs, while mid-gut and terminal abdominal ganglion (TAG) maintained only *Gb'per* mRNA rhythm and anterior stomach lost both rhythms. These data suggest that the rhythms outside the optic lobe are controlled by the optic lobe to different degrees, and that the oscillatory mechanism may be different from that of *Drosophila*.

4.3. Materials and methods

Animals

Adult male crickets, *G. bimaculatus*, were used. They were purchased or obtained from a laboratory colony maintained under standard environmental conditions with a light dark (LD) cycle of 12 h light to12 h dark (light: 06:00–18:00 h; Japanese standard time) at a constant temperature of 25 ± 0.5 ^{o}C. They were fed laboratory chow and water.

RNA preparation and reverse transcription

Total RNA was extracted and purified from 5 adult male brains, anterior stomachs, mid-guts, MTs, testes and 10 adult male terminal abdominal ganglia (TAG) with ISOGEN (Nippon Gene, Tokyo, Japan) or TRIzol (Invitrogen, Carlsbad, CA). To remove contaminating genomic DNA, total RNA was treated with DNase I. About 500 ng of total RNA of each sample was reverse transcribed with random 6mers using Primescript™ RT reagent kit (Takara, Ohtsu, Japan).

Detection of mRNAs in the neural and peripheral tissues

RT-PCR was used to investigate whether clock genes were expressed in the male brain, anterior stomach, mid-gut, Malpighian tubule, testis and TAG. PCR was performed with Gb-*per*-F1 (5′-AAGCAAGCAAGCATCCTCAT-3′) and Gb-*per*-R1 (5′-CTGAGAAAGGAGGCCACAAG-3′) primers for *Gb'per* (GenBank/EMBL/DDBJ accession no. AB375516), Gb-*tim*-F1 (5′-GCGGTATTTAGAAGGCCACACAGTAA-3′) and Gb-*tim*-A2048 (5′-CAGCTCCAATTCCAGTTGTG-3′) for *Gb'tim* (GenBank/EMBL/DDBJ accession no. AB548625; Danbara et al., 2010), and Gb-*rpl18a*-195F (5′-GCTCCGGATTACATCGTTGC-3′) and Gb- *rpl18a*-339R (5′-GCCAAATGCCGAAGTTCTTG-3′) for *Gb'rpl18a* (Gen- Bank/EMBL/DDBJ accession no. DC448653), a housekeeping gene. PCR amplifications of the synthesized cDNA were performed for 30 cycles according to the following schedule: 94 °C for 30 s, 63.8 °C for 30 s, and 72 °C for 1 min for *Gb'per*, 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 2 min for *Gb'tim*, and 94 °C for 30 s, 66 °C for 30

s, and 72 ºC for 1 min for *Gb'rpl18a*. The PCR products were analyzed by electrophoresis. The amounts of PCR products detected were quantified by ImageJ (http://rsb.info.nih.gov/nih-image/).

Measurement of mRNA levels

Quantitative real-timeRT-PCR (qPCR) was performed to measure mRNA levels byMx3000P real-time PCR System (Stratagene, La Jolla, CA) using FastStart Universal SYBR Green Master (Roche, Tokyo, Japan) including SYBR Green with Gb-*per*-F1 and Gb-*per*-R1 primers for *Gb'per*, Gb-*tim*-A1 (5'-CATCCGTTGACCCTATTTGC-3') and Gb-*tim*-S1 (5'-GACGAATGCATTTGGTTGTG-3') for *Gb'tim*, Gb-*rpl18a*- 195F and Gb-*rpl18a*-339R for *Gb'rpl18a*. Standard curves for the transcripts were generated by serial (10×) dilutions of amplified cDNAs and included in each real-time PCR run. After 40 cycles, samples were run for melting curve analysis, and in every case, a single expected amplicon was confirmed. The results were analyzed using software associated with the instrument. The values were normalized with the values of *Gb'rpl18a* at each time point as described previously (Moriyama et al., 2008, 2009). Four independent experiments were used to calculate the mean ± SEM.

Surgical operation

Animals were collected from the colony chamber between 10:00 (Zeitgeber time (ZT) 4; ZT0 corresponds to light-on and ZT12 corresponds to light-off) and 14:00 (ZT8) for operation. The outer two neuropils of the optic lobe, the lamina and medulla (hereafter referred to as the ''optic lobe''), were

surgically removed as follows. An incision was made along the margin of the compound eye, and the eye capsule was pried open so that the optic lobe and optic nerves were exposed. Then the optic stalk and optic nerves were cut with a pair of fine scissors to remove the optic lobe. The eye capsule was then put back into the original position. The operation was made under CO_2 anesthesia. The whole procedure took about 5min. The post-operative animal was kept in a plastic container and placed in the rearing room under LD 12:12 at 25 ^{o}C until use.

Statistical analysis

Significance of the daily and circadian rhythmicity in mRNA expression was examined and the acrophase was determined by the single cosiner method (Nelson et al., 1979) fitting a cosine curve of 24 h period using Time Series Analysis Serial Cosiner 6.3 (Expert Soft Technologie Inc.). For DD experiments, data starting at CT2 on the first day of DD were used for analysis.

4.4. Results

Expression of *Gb'per* and *Gb'tim* mRNA in tissues outside the optic lobe

Expression of *Gb'per* and *Gb'tim* mRNA was first examined in tissues outside the optic lobe. Total RNAs were extracted from the brain, TAG, testis, mid-gut, anterior stomach, and MTs, reverse transcribed and used for RT-PCR. As shown in Fig. 4-1, mRNAs of both *Gb'per* and *Gb'tim* were detected in all the examined tissues. For *Gb'per*, a relatively high amount of mRNA was detected in the brain, TAG, mid-gut and MTs and the least was detected in testis; the

amount of *Gb'tim* mRNA was greatest in the MTs and least in the testis.

To investigate whether *Gb'per* and *Gb'tim* transcripts oscillated in nervous tissues, levels of *Gb'per* and *Gb'tim* mRNAs were examined in the brain and TAG under 12 h light to 12 h dark (LD) cycle and the ensuing DD by qPCR (Fig. 4-2). Both *Gb'per* and *Gb'tim* showed a daily rhythm of mRNA expression with a peak during the late day to early night under LD in the brain (Cosiner, $P < 0.001$). In TAG, although significant rhythmicity was detected only in *Gb'tim* (Cosiner, $P < 0.001$), both *Gb'per* and *Gb'tim* showed a rhythmic profile in LD. The amplitude was nearly 2-fold for both *Gb'per* and *Gb'tim* in the brain, while it was about 1.9-fold for *Gb'tim* in TAG. The mRNA expression rhythm persisted in the ensuing DD with a peak during the early subjective night for at least two cycles (Cosiner, $P < 0.01$), suggesting that it is driven by an endogenous mechanism. In the brain, however, the mRNA expression of *Gb'tim* was rather quickly dampened in DD (Fig. 4-2A). The rhythmic profiles were basically similar to those in the optic lobe (Moriyama et al., 2008), the central clock tissue of the cricket (Tomioka and Chiba, 1984, 1992).

Then expression patterns of *Gb'per* and *Gb'tim* mRNAs were examined in peripheral tissues by qPCR with total RNAs extracted from 4 different peripheral tissues: anterior stomach, mid-gut, testis, and MTs (Fig. 4-3). In the anterior stomach, although both *Gb'per* and *Gb'tim* showed a weakly rhythmic expression profile with a peak during mid- to late-night in LD, single cosiner analysis revealed a statistically significant rhythm only for *Gb'tim* (Cosiner, $P < 0.01$; Fig. 4-3C). In the ensuing DD, however, they both showed robust rhythms with a peak during the subjective midnight (Cosiner, $P < 0.001$; Fig. 4-3C). The

amplitude for *Gb'per* and *Gb'tim* was about 1.3-fold and about 1.4-fold, respectively, in LD and about 1.6-fold and about 2.2-fold, respectively, in DD. In the mid-gut, significant rhythmicity was detected in *Gb'tim* mRNA expression in both LD and DD (Cosiner, $P < 0.01$) although its level was gradually reduced (Fig. 4-3D); the *Gb'per* mRNA also showed a weakly rhythmic profile in both LD and DD, but it was revealed to be significant only in DD (Cosiner, $P < 0.03$; Fig. 4-3D). The amplitude for *Gb'per* and *Gb'tim* was about 1.2-fold and about 1.4-fold, respectively, in LD and about 1.2-fold and about 1.5-fold, respectively, in DD. No clear rhythms of *Gb'per* and *Gb'tim* expression were found in the testis and MTs in LD (Cosiner, $P > 0.05$; Fig. 4-3A and B). In the testis, however, both *Gb'per* and *Gb'tim* showed a rhythmic profile peaking in the early subjective night in DD (Cosiner, $P < 0.05$ for *Gb'per* and $P < 0.01$ for *Gb'tim*). The amplitudes of *Gb'per* and *Gb'tim* were approximately 1.5-fold and 1.4-fold, respectively.

Effects of optic lobe removal on mRNA rhythms outside the optic lobe

To examine whether the mRNA rhythms were driven by the central pacemaker in the optic lobe, levels of *Gb'per* and *Gb'tim* mRNA were measured by qPCR in the brain, TAG, anterior stomach and mid-gut of the adult male crickets with the optic lobes bilaterally removed. The tissue sampling was performed every 4 h under LD, 25 ^{o}C, starting at ZT14, at least 48 h after the operation. In the brain, both *Gb'per* and *Gb'tim* showed a robust rhythmic profile of mRNA expression (Cosiner, $P < 0.001$ for *Gb'per* and $P < 0.002$ for *Gb'tim*; Fig. 4-4A). The peak of *Gb'tim* mRNAs occurred approximately 4 h earlier than that in intact crickets, however. This was confirmed by analysis

with the single cosiner method; the acrophase was ZT12.9 and ZT16.2 for the optic lobeless and intact crickets, respectively. Analysis with the single cosiner method also revealed that the phase of *Gb'per* mRNA expression was advanced by nearly 2 h (ZT13.3 and ZT15.4 for optic lobeless and intact crickets, respectively). In the TAG and mid-gut, *Gb'per* clearly showed rhythmic mRNA expression (Cosiner, $P < 0.05$ for TAG and $P < 0.001$ for mid-gut), while surprisingly *Gb'tim* mRNA showed no clear oscillation (Figs. 4-4B and 4-5B, Cosiner, $P > 0.4$); the *Gb'tim* levels were up-regulated and down-regulated, respectively, in the TAG and mid-gut. No phase changes associated with the optic lobe removal in *Gb'per* rhythms were observed in either the TAG or mid-gut. In the anterior stomach, both *Gb'per* and *Gb'tim* exhibited a substantial change but no daily rhythmic mRNA expression (Fig. 4-5A, Cosiner, $P > 0.5$ for *Gb'per* and $P > 0.1$ for *Gb'tim*).

4.5. Discussion

The results of the present study clearly indicate that there are daily and circadian oscillations in tissues beyond the optic lobes. It has been reported that circadian oscillations exist in various tissues of insects. For example, *per* is rhythmically expressed in cells of various tissues of *Drosophila* including the compound eye, antennae, proboscis, wings, legs, ring gland and MTs (Plautz et al., 1997; Emery et al., 1997; Giebultowicz and Hege, 1997). In moths, sperm release from testis to the associated vas deferens as well as ecdysone secretion from the prothoracic gland is controlled in a circadian manner by peripheral oscillators (see Giebultowicz, 1999). These peripheral oscillations probably have

tissue specific roles. In the cricket, since the TAG innervates the reproductive system including the accessory glands and associated tissues (Kumashiro and Sakai, 2001), it may be involved in regulation of circadian reproductive rhythms (Loher, 1974). The rhythm of anterior stomach and mid-gut may be related to digestive function as has been suggested in vertebrate digestive systems (LeSauter et al., 2009). Unlike in *Drosophila*, however, the cricket's MTs showed no clear rhythmic profile in *Gb'per* and *Gb'tim* expression in LD (Fig. 4-3B). These data suggest that the circadian regulation is tissue specific and sometimes species specific. The specificity may be related to their functional role in respective temporal organizations of animals.

In LD cycles, no significant oscillation was revealed for *Gb'per* mRNA levels in TAG, anterior stomach and mid-gut and for either *Gb'per* or *Gb'tim* in testes by the single cosiner method, while in DD, all of these tissues showed significant oscillations (Figs. 4-2A and 4-3). Although the reason for this is unclear, light might have a direct effect on clock gene expression in these tissues as *Drosophila* peripheral tissues have their own photoreceptors (Plautz et al., 1997; Ito et al., 2008).

The present study revealed that the mRNA expression rhythm persisted after optic lobe removal in the brain, TAG and mid-gut, while the anterior stomach lost its rhythm (Figs. 4-4 and 4-5). This fact suggests that regulation by the optic lobe pacemaker is tissue specific. The rhythm persistence in tissues outside the optic lobe is in parallel with findings in *Drosophila* and vertebrates, where some peripheral tissues possess a self-sustaining circadian oscillator (Plautz et al., 1997; Schibler, 2009). In *Drosophila*, rhythmic expression of *per* in

the peripheral tissues including MTs continued *in vitro* and was able to entrain to the light–dark regime of the culture condition (Plautz et al., 1997; Emery et al., 1997; Giebultowicz et al., 2000). Transplanted MTs maintained their original phase of oscillation even in host flies which had been entrained to the reverse light–dark cycle of the donor (Giebultowicz et al., 2000). Thus these peripheral oscillators seem to function as stand-alone pacemakers. The same is true for the olfaction rhythm as an output of the antennal clock (Tanoue et al., 2008). The present study has not examined rhythms in culture conditions, but their persistence after removal of the optic lobe suggests that the rhythmic tissues of the cricket possess a self-sustaining circadian oscillator. However, it is premature to totally exclude a possibility that a circadian oscillator outside the optic lobe regulates those tissues.

The mRNA oscillation in the brain persisted even 48 h after the optic lobe was removed (Fig. 4-4A), indicating that the brain contains an oscillator independent of the central pacemaker. The finding is consistent with earlier reports that crickets often show residual locomotor rhythms (Tomioka, 1985; Stengl, 1995) after optic lobe removal or exhibit rhythms entrained to temperature cycles or light cycles (Rence and Loher, 1975; Tomioka and Chiba, 1989). However, the phase of the brain mRNA rhythms was advanced by 4 h after optic lobe removal (Fig. 4-4A). The phase advance may be explained by a free-running *period* shorter than 24 h, but it seems more likely that the phase was regulated by the clock in the optic lobe. This is because the free-running period is normally slightly shorter than 24 h (Tomioka and Chiba, 1982b), and the expected magnitude of phase advance would be only 0.2 h after 48 h of

free-running. Considering no apparent phase change was induced by optic lobe removal in the *Gb'per* rhythm of the mid-gut and TAG (Figs. 4-4B and 4-5B), the brain mRNA rhythms are most likely phase regulated by the central pacemaker in the optic lobe. These results again suggest that the rhythm is controlled by the optic lobe pacemaker in a tissue specific manner.

However, the anterior stomach lost its rhythm after optic lobe removal. This fact suggests that oscillation in this tissue is probably dependent on the central pacemaker in the optic lobe. A similar observation has been reported for the olfactory rhythm in cockroach antennae, where electroanntenographic (EAG) rhythms were lost after optic lobe removal (Page and Koelling, 2003). In the cockroach antenna, however, individual olfactory receptor neurons are known to exhibit circadian rhythms independent of the central pacemaker (Saifullah and Page, 2009). Thus, it may be possible that individual cells in the anterior stomach possess the rhythm but without the central pacemaker they lose phase synchrony, as seen in some mammalian peripheral tissues (Schibler, 2009).

More interestingly, optic lobe removal differentially affected the *Gb'per* and *Gb'tim* mRNA expression rhythms. In the brain mRNA levels of *Gb'per* increased nearly 2-fold while *Gb'tim* remained at the same level (Fig. 4-4A). In the TAG and mid-gut, optic lobe removal eliminated rhythmic expression of only *Gb'tim*; *Gb'per* mRNA maintained the rhythmic profile (Figs. 4-4B and 4-5B). These results suggest that the optic lobe regulates rhythmic expression of *Gb'tim* but not of *Gb'per*, suggesting the existence of different oscillatory mechanisms for *Gb'per* and *Gb'tim*. The persistence of *Gb'per* mRNA rhythm

without *Gb'tim* oscillation in TAG and mid-gut suggests that the rhythmic expression of *Gb'per* does not require the oscillation of *Gb'tim*. This is inconsistent with *Drosophila*'s oscillatory model where PER and TIM cooperate to form a negative feedback loop for their rhythmic expression (Stanewsky, 2002; Hardin, 2006). The *per* oscillation without *tim* is supported by recent findings in honeybees and butterflies. In honeybees *tim* does not exist in the genome (Rubin et al., 2006), and vertebrate-type CRYPTOCHROME (CRY) interacts with PER to form a negative feedback loop in the monarch butterfly (Zhu et al., 2008).

The role of *Gb'per* and *Gb'tim* was have examined in the cricket central clock using RNAi. Knocking-down of *Gb'per* and *Gb'tim* by double stranded RNA (dsRNA) of each of the genes significantly suppresses expression of their mRNA (Moriyama et al., 2008; Danbara et al., 2010). But ds*Gb'per* and ds*Gb'tim* have different effects on the circadian locomotor rhythm; ds*Gb'per* totally abolished the rhythm (Moriyama et al., 2008, 2009) while ds*Gb'tim* has little effect (Danbara et al., 2010). These facts also suggest that *Gb'per* and *Gb'tim* play different roles in the cricket circadian clock. But it should be noticed that the peripheral oscillatory mechanism may be different from that of the central pacemaker, since it is known that in *Drosophila* there is a fundamental difference between peripheral and central oscillators. The most remarkable difference is a function of CRY. Flies carrying cry^{b}, a loss-of-function mutation of *cryptochrome* gene, showed intact molecular oscillations of PER and TIM in the central pacemaker neurons but lost molecular oscillation in the MTs (Ivanchenko et al., 2001). In antennae, the rhythmic EAG responses to odorants were also

abolished in the mutant (Krishnan et al., 2001). These results indicate that CRY functions as a core component in these peripheral oscillators, while it only functions as a photoreceptor in the central pacemaker neurons (Emery et al., 2000). Future study on the role of not only *Gb'per* and *Gb'tim* but also other clock genes including *cry* are needed to reveal the molecular machinery of the peripheral circadian clock in the cricket.

References

Balkenohl, M., Weber, F. (1981) Sind auch bei holometabolen Insekten circadiane Schrittmacher der Aktivitat in den optischen Ganglion lokalisiert? Mitteilungen der Deutschen Gesellschaft für Allgemeine und Angewandte Entomologie 3, 223-227.

Danbara, Y., Sakamoto, T., Uryu, O., Tomioka, K. (2010) RNA interference of timeless gene does not disrupt circadian locomotor rhythms in the cricket *Gryllus bimaculatus*. Journal of Insect Physiology 56, 1738–1745.

Emery, I. F., Noveral, J. M., Jamison, C. F., Siwickii, K. K. (1997) Rhythms of *Drosophila period* gene in culture. Proceedings of the National Academy of Sciences USA 94, 4092-4096.

Emery, P., Stanewsky, R., Hall, J. C., Rosbash, M. (2000) *Drosophila cryptochromes*: a unique circadian-rhythm photoreceptor. Nature 404, 456-457.

Giebultowicz, J. M. (1999) Insect circadian clocks: is it all in their heads? Journal of Insect Physiology 45, 791-800.

Giebultowicz, J. M., Hege, D. M. (1997) Circadian clock in Malpighian tubules. Nature 386, 664.

Giebultowicz, J. M., Riemann, J. G., Raina, A. K., Ridgway, R. L. (1989) Circadian system controlling release of sperm in the insect testes. Science 245, 1098-1100.

Giebultowicz, J. W., Stanewsky, R., Hall, J. C., Hege, D. M. (2000) Transplanted *Drosophila* excretory tubules maintain circadian clock cycling out of phase with the host. Current Biology 10, 107-110.

Handler, A. M., Konopka, R. J. (1979) Transplantation of a circadian pacemaker in *Drosophila*. Nature 279, 236-238.

Hardin, P. E. (2006) Essential and expendable features of the circadian timekeeping mechanism. Current Opinion in Neurobiology 16, 686-692.

Ito, C., Goto, S. G., Shiga, S., Tomioka, K., Numata, H. (2008) Peripheral

circadian clock for the cuticle deposition rhythm in *Drosophila melanogaster*. Proceedings of the National Academy of Sciences USA 105, 8446-8451.

Ivanchenko, M., Stanewsky, R., Giebultowicz, J. M. (2001) Circadian photoreception in *Drosophila*: functions of cryptochrome in peripheral and central clocks. Journal of Biological Rhythms 16, 205-215.

Kasai, M., Chiba, Y. (1987) Effects of optic lobe ablation on circadian activity in the mosquito, *Culex pipiens pallens*. Physiological Entomology 12, 59-65.

Krishnan, B., Levine, J. D., Lynch, M. K., Dowse, H. B., Funes, P., Hall, J. C., Hardin, P. E., Dryer, S. E. (2001) A new role for cryptochrome in a *Drosophila* circadian oscillator. Nature 411, 313-317.

Kumashiro, M., Sakai, M. (2001) Reproductive behaviour in the male cricket *Gryllus bimaculatus* DeGeer. I. Structure and function of the genitalia. Journal of Experimental Biology 204, 1123-1137.

LeSauter, J., Hoque, N., Weintraub, M., Pfaff, D. W., Silver, R. (2009) Stomach ghrelin-secreting cells as food-entrainable circadian clocks. Proceedings of the National Acadamy of Sciences USA 106, 13582-13587.

Loher, W. (1974) Circadian control of spermatophore formation in the cricket *Teleogryllus commodus* Walker. Journal of Insect Physiology 20, 1155-1172.

Moriyama, Y., Sakamoto, T., Matsumoto, A., Noji, S., Tomioka, K. (2009) Functional analysis of the circadian clock gene *period* by RNA interference in nymphal crickets *Gryllus bimaculatus*. Journal of Insect Physiology 55, 396-400.

Moriyama, Y., Sakamoto, T., Karpova, S. G., Matsumoto, A., Noji, S., Tomioka, K. (2008) RNA interference of the clock gene *period* disrupts circadian rhythms in the cricket *Gryllus bimaculatus*. Journal of Biological Rhythms 23, 308-318.

Page, T. L. (1982) Transplantation of the cockroach circadian pacemaker. Science 216, 73-75.

Page, T. L., Koelling, E. (2003) Circadian rhythm in olfactory response in the antennae controlled by the optic lobe in the cockroach. Journal of Insect Physiology 49, 697-707.

Plautz, J. D., Kaneko, M., Hall, J. C., Kay, S. A. (1997) Independent photoreceptive circadian clocks throughout *Drosophila*. Science 278, 1632-1635.

Rence, B. G., Loher, W. (1975) Arrhythmically singing crickets: thermoperiodic reentrainment after bilobectomy. Science 190, 385-387.

Rubin, E. B., Shemesh, Y., Cohen, M., Elgavish, S., Robertson, H. M., Bloch, G. (2006) Molecular and phylogenetic analyses reveal mammalian-like clockwork in the honey bee (*Apis mellifera*) and shed new light on the molecular evolution of the circadian clock. Genome Research 16, 1352-1365.

Saifullah, A. S. M., Page, T. L. (2009) Circadian regulation of olfactory receptor neurons in the cockroach antenna. Journal of Biological Rhythms 24,

144-152.
Saunders, D. S. (2002) Insect Clocks, 3rd Edition. Amsterdam: Elsevier.
Schibler, U. (2009) The 2008 Pittendrigh/Aschoff lecture: Peripheral phase coordination in the mammalian circadian timing system. Journal of Biological Rhythms 24, 3-15.
Stanewsky, R. (2002) Clock mechanisms in *Drosophila*. Cell and Tissue Research 309, 11-26.
Stengl, M. (1995) Pigment-dispersing hormone-immunoreactive fibers persist in crickets which remain rhythmic after bilateral transection of the optic stalks. Journal of Comparative Physiology A 176, 217-228.
Tanoue, S., Krishnan, P., Chatterjee, A., Hardin, P. E. (2008) G protein-coupled receptor kinase 2 is required for rhythmic olfactory responses in *Drosophila*. Current Biology 18, 787-794.
Tomioka, K. (1985) Residual circadian rhythmicity after bilateral lamina-medulla removal or optic stalk transection in the cricket, *Gryllus bimaculatus*. Journal of Insect Physiology 31, 653-657.
Tomioka, K., Chiba, Y. (1982a) Post-embryonic development of circadian rhythm in the cricket, *Gryllus bimaculatus*. Journal of Comparative Physiology A 147, 299-304.
Tomioka, K., Chiba, Y. (1982b) Persistence of circadian ERG rhythms in the cricket with optic tract severed. Naturwissenschaften 69, 355-356.
Tomioka, K., Chiba, Y. (1984) Effects of nymphal stage optic nerve severance or optic lobe removal on the circadian locomotor rhythm of the cricket, *Gryllus bimaculatus*. Zoological Science 1, 385-394.
Tomioka, K., Chiba, Y. (1989) Photoperiodic entrainment of locomotor activity in crickets (*Gryllus bimaculatus*) lacking the optic lobe pacemaker. Journal of Insect Physiology 35, 827-835.
Tomioka, K., Chiba, Y. (1992) Characterization of optic lobe circadian pacemaker by *in situ* and *in vitro* recording of neuronal activity in the cricket *Gryllus bimaculatus*. Journal of Comparative Physiology A 171, 1-7.
Truman, J. W. (1974) Physiology of insect rhythms. IV. Role of the brain in the regulation of the flight rhythm of the giant silkmoths. Journal of Comparative Physiology A 95, 281-296.
Wiedenmann, G., Lukat, R., Weber, F. (1986) Cyclic layer deposition in the cockroach endocuticle: a circadian rhythm? Journal of Insect Physiology 32, 1019-1027.
Wills, S. A., Page, T. L., Colwell, C. S. (1985) Circadian rhythms in the electroretinogram of the cockroach. Journal of Biological Rhythms 1, 25-37.
Zhu, H., Sauman, I., Yuan, Q., Casselman, A., Emery-Le, M., Emery, P., Reppert, S. M. (2008) Cryptochromes define a novel circadian clock mechanism in monarch butterflies that may underlie sun compass navigation. PLoS Biology 6, 138-155.

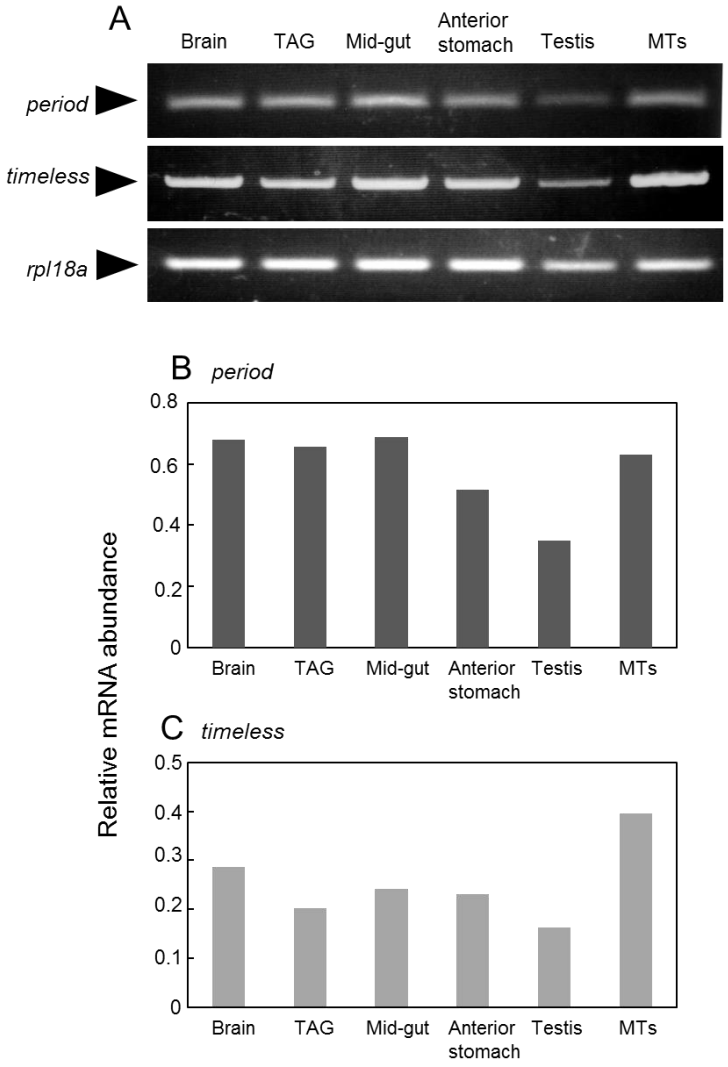

Fig. 4-1. Expression of *Gb'per* and *Gb'tim* mRNA in the adult brain, terminal abdominal ganglion (TAG), testis, Malpighian tubules (MTs), anterior stomach, and mid-gut of the cricket *Gryllus bimaculatus* kept under LD 12:12, 25 ºC.

(A) RT-PCR was performed with 22.5 ng total RNA extracted from the tissues at ZT14. (B) and (C) show relative amount of *Gb'per* (B) and *Gb'tim* (C) mRNA in each tissue to that of *Gb'rpl18a* mRNA.

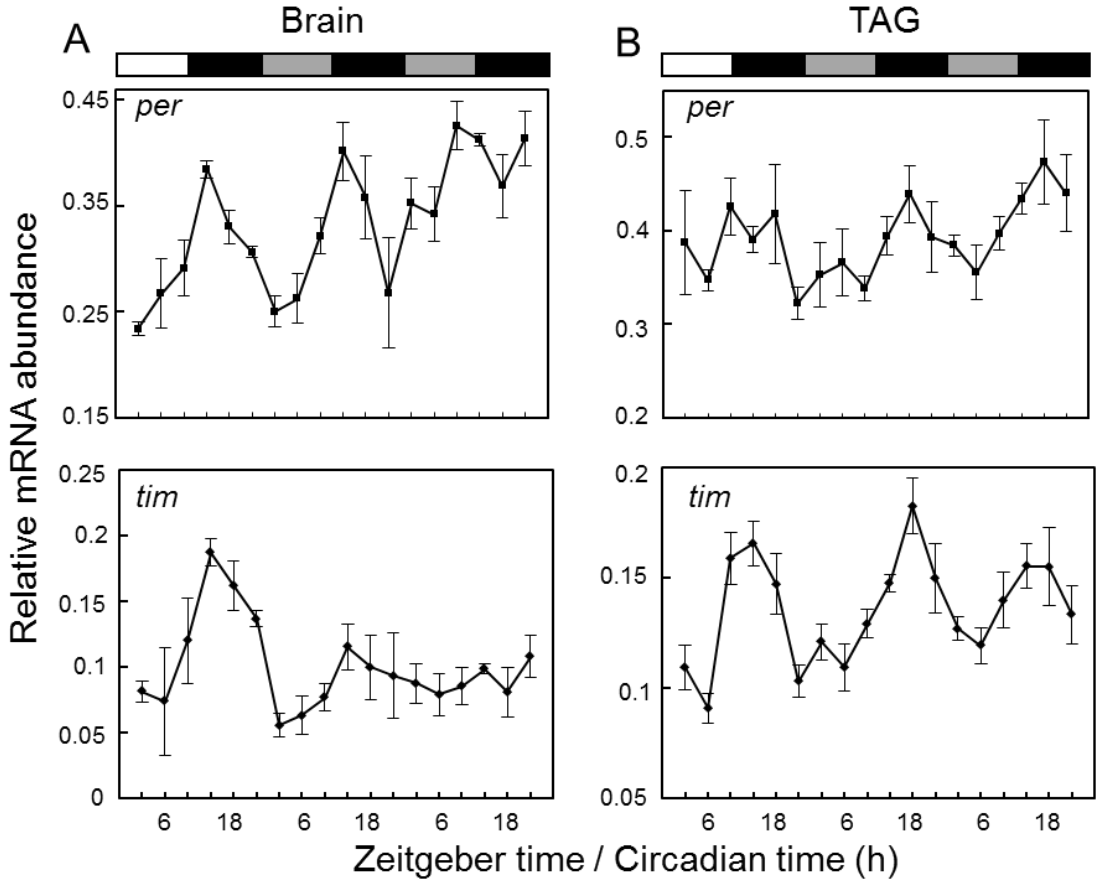

Fig. 4-2. Transcripts abundance over time in the cricket brain and terminal abdominal ganglion (TAG) under LD, 25 °C and the ensuing DD.

(A) *Gb'per* and *Gb'tim* in brain; (B) *Gb'per* and *Gb'tim* in TAG. The abundance of *Gb'per* and *Gb'tim* mRNA was measured by quantitative real-time RT-PCR with total RNA extracted from the tissues of adult crickets that were collected at 4 h intervals starting at 2 h after light-on (ZT2). The value shown is relative to the amount of *Gb'rpl18a* mRNA. Vertical bars indicate SEM. Black, white and gray bars indicate night/subjective night, day and subjective day, respectively. Note the mRNA levels of both *Gb'per* and *Gb'tim* show rhythmic expression with a peak in the early night or subjective night.

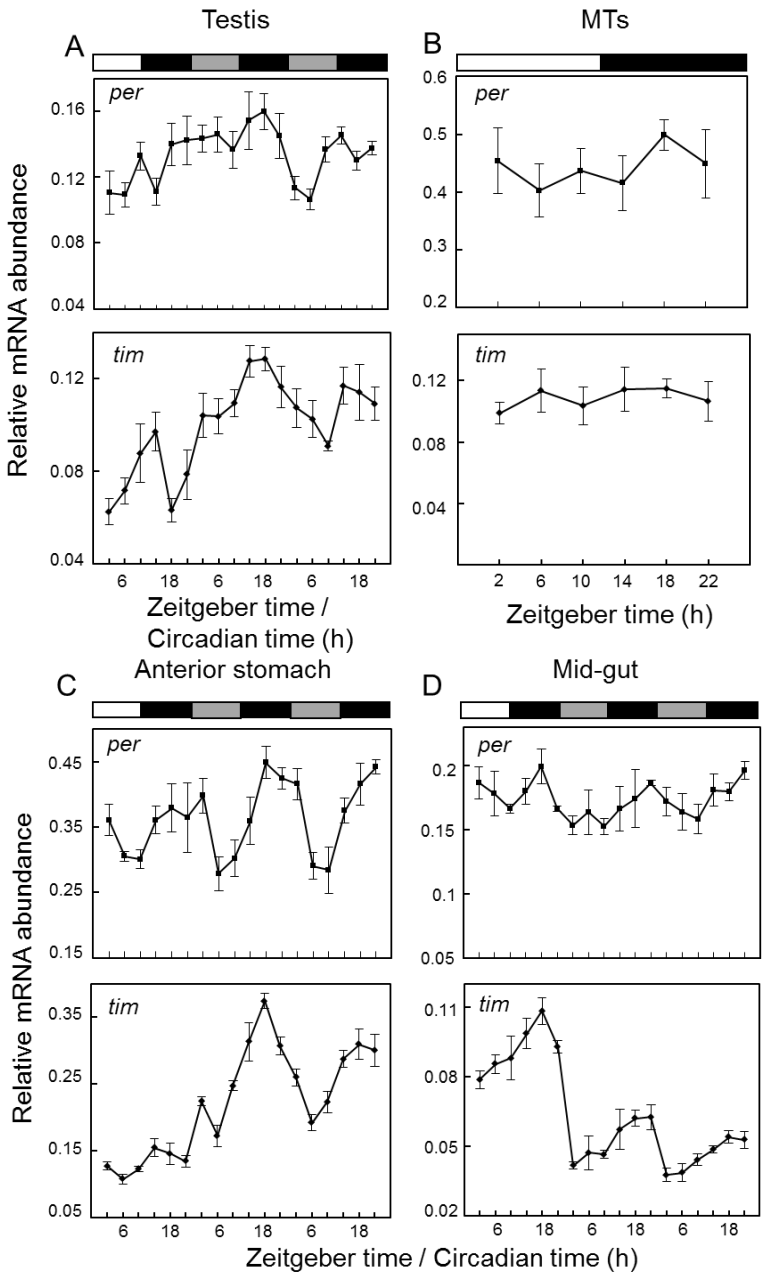

Fig. 4-3. Transcripts abundance of *Gb'per* and *Gb'tim* over time in the cricket testis (A), MTs (B), anterior stomach (C), and mid-gut (D) under LD and the ensuing DD.

The abundance of *Gb'per* and *Gb'tim* mRNA was measured by quantitative real-time RT-PCR with total RNA extracted from the tissues of adult crickets that were collected at 4 h intervals starting at 2 h after light-on (ZT2). The value shown is relative to the amount of *Gb'rpl18a* mRNA. Vertical bars indicate SEM. Black, white and gray bars indicate night/subjective night, day and subjective day, respectively. MTs showed no clear rhythm while testis, anterior stomach and mid-gut showed a weak but significant rhythm in either or both *Gb'per* and *Gb'tim* mRNA levels. For further explanations see text.

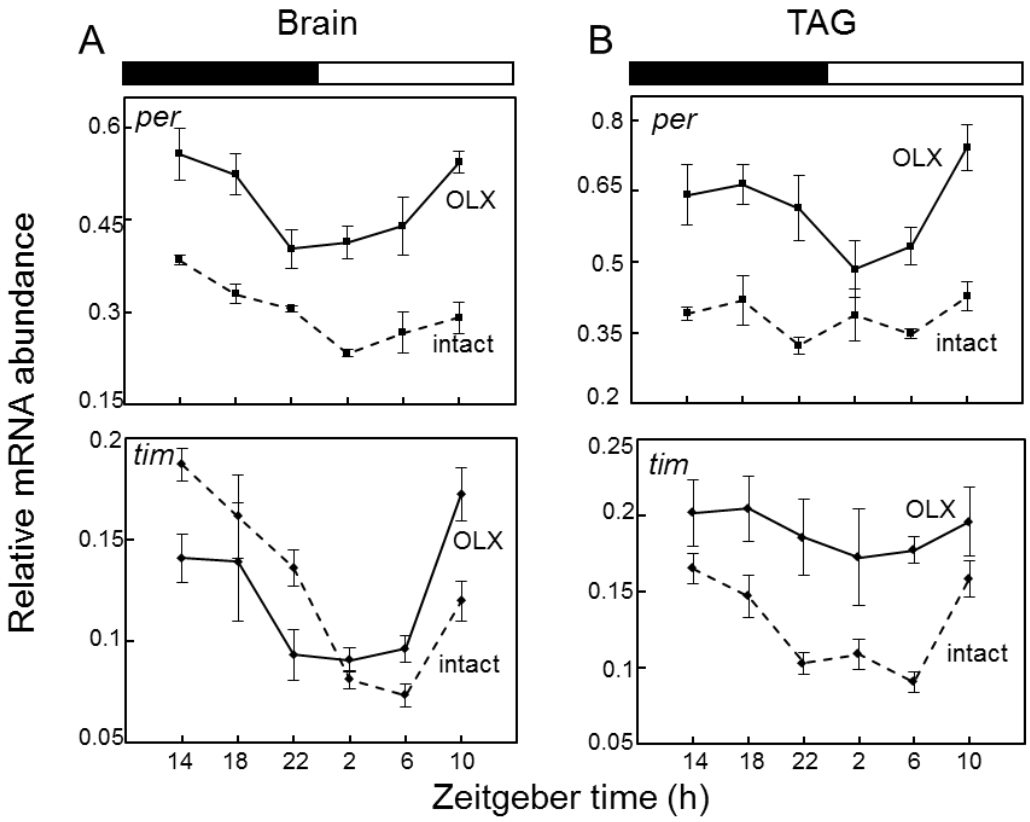

Fig. 4-4. Transcripts abundance over time in cricket brain (A) and terminal abdominal ganglion (TAG, B) under LD 48 h after the optic lobe was bilaterally removed.

Solid lines are for the crickets receiving the optic lobe removal (OLX) and broken lines for intact animals duplicated from Fig. 4-2. The abundance of *Gb'per* and *Gb'tim* mRNA was measured by quantitative real-time RT-PCR with total RNA extracted from tissues of adult crickets that were collected at 4 h intervals starting at 2 h after light-on (ZT2). The values shown are relative to the amount of *Gb'rpl18a* mRNA. Vertical bars indicate SEM. Black and white bars indicate night and day, respectively. In the brain both *Gb'per* and *Gb'tim* show rhythmic expression with a peak advanced by 4 h relative to that of intact crickets. In TAG, only *Gb'per* shows a rhythm after optic lobe removal.

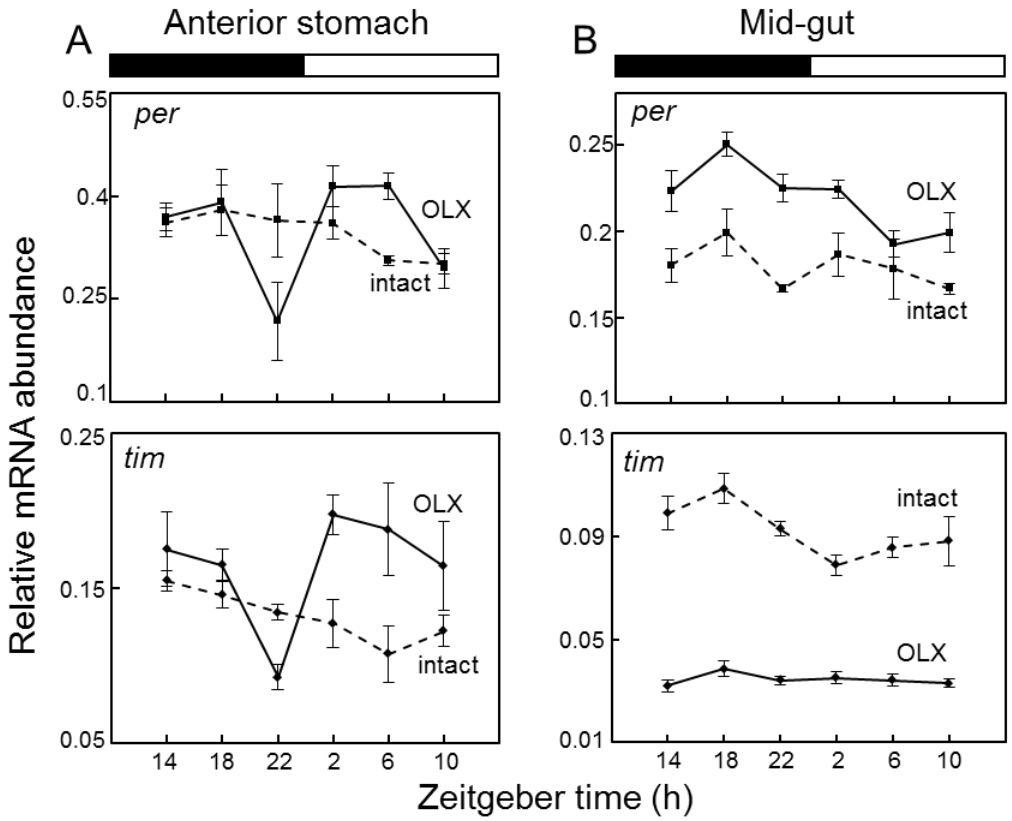

Fig. 4-5. Transcripts abundance over time in cricket anterior stomach (A) and mid-gut (B) under LD 48 h after the optic lobe was bilaterally removed.
Solid lines are for the crickets receiving the optic lobe removal (OLX) and broken lines for intact animals duplicated from Fig. 4-3. The abundance of *Gb'per* and *Gb'tim* mRNA was measured by quantitative real-time RT-PCR with total RNA extracted from tissues of adult crickets that were collected at 4 h intervals starting at 2 h after light-on (ZT2). The values shown are relative to the amount of *Gb'rpl18a* mRNA. Vertical bars indicate SEM. Black and white bars indicate night and day, respectively. Anterior stomach showed no clear rhythm after optic lobe removal, while mid-gut showed a rhythm only in *Gb'per* with a similar phase but at a slightly higher level than observed in intact crickets. The *Gb'tim* expression in the mid-gut became arrhythmic and the mRNA levels were significantly lowered after optic lobe removal.

Chapter 5.

Post-embryonic development of the circadian oscillations within and outside the optic lobe in the cricket, *Gryllus bimaculatus*

5.1. Abstract

The adult cricket *Gryllus bimaculatus* has a central clock in the optic lobe that regulates overt activity rhythms and secondary oscillators in the tissue outside the optic lobe. Here properties of the rhythmic expression of clock genes were investigated in the optic lobe and extra-optic lobe tissues in nymphs, and compared them with those of adults. In the optic lobe, mRNA of the clock genes, *period* (*per*), *timeless* (*tim*), *cycle* (*cyc*) and *Clock* (*Clk*) were expressed in patterns similar to those in adult profiles, but at significantly lower levels. Among the extra-optic lobe tissues, the brain and TAG showed a rhythmic expression of *per* and *tim,* the mid-gut only in *tim,* and the anterior-stomach in none of the genes studied. The mRNA levels of clock genes were again significantly lower than those in adults. Unlike in adults, the brain and mid-gut lost their rhythms of clock gene expression in DD, and when the optic lobes were bilaterally removed. These results suggest that the rhythms outside the optic lobe are weak in nymphs, and may become robust after the imaginal molt.

5.2. Introduction

In insects, many physiological functions, such as eclosion (Myers et al., 2003), cuticle formation (Ito et al., 2008) and activity of salivary glands (Weitzel and Rensing, 1981), follow a daily rhythmic pattern. These daily rhythms are controlled by an endogenous mechanism called the circadian system, which consists of the central clock and peripheral oscillators (Pittendrigh, 1974). The central clock regulates overt activity rhythms and has been shown to reside in the optic lobe in crickets, cockroaches, and beetles (Tomioka and Chiba, 1992; Page, 1982; Balkenohl and Weber, 1981), and in the central brain in flies, mosquitoes, and moths (Handler and Konopka, 1979; Kasai and Chiba, 1987; Truman, 1974). In addition to the central clock, many peripheral tissues also harbor a circadian oscillator as has been shown by clock gene reporter

expression in *Drosophila* (Hege et al., 1997; Plautz et al., 1997).

It has been recently shown that tissues outside the optic lobe also show rhythms of clock gene expression in the cricket *G. bimaculatus* (Chapter 4). This cricket shows a phase reversal from a nymphal diurnal locomotor activity rhythm to an adult nocturnal rhythm (Tomioka and Chiba, 1982). The circadian clock in the optic lobe has been shown to have common physiological properties in both nymphs and adults (Tomioka and Chiba, 1992), suggesting that changes outside the optic lobe are related to phase reversal in overt activity rhythms. This study was thus designed to investigate circadian rhythms in tissues outside the optic lobe in the adult and nymphal crickets using mRNA expression of the clock genes, *period* (*per*), *timeless* (*tim*), *cycle* (*cyc*) and *Clock* (*Clk*), which are major players in the oscillatory mechanism in insects (cf. Stanewsky, 2002; Hardin, 2006; Yuan et al., 2007; Vitaterna et al., 1999). Some nymphal tissues were found to show rhythmic expression of the clock genes although their expression levels were significantly lower than in adults. Unlike in adults, the brain and mid-gut lost their rhythms when transferred to DD or the optic lobes were bilaterally removed. These results suggest that the oscillations of the nymphal extra-optic lobe tissues are weak and may become robust after the imaginal molt.

5.3. Materials and methods

Animals

Final (8^{th}) instar (nymphal) and adult male crickets, *G. bimaculatus*, were used. They were purchased or obtained from our laboratory colony maintained under standard environmental conditions with a light dark (LD) cycle of 12 h light to12 h dark (light: 06:00–18:00 h; Japanese standard time) at a constant temperature of 25 ± 0.5˚C. They were fed with laboratory chow and water.

RNA preparation and reverse transcription

Tissue samplings of optic lobes, brains, terminal abdominal ganglia (TAG), anterior stomachs, and mid-guts were performed under dissecting microscope and dim red light was used during dark period. Total RNA was extracted and purified from the tissues with TRIzol reagent (Invitrogen, Carlsbad, CA). The number of tissues was 6 and 10 for the optic lobe and TAG, respectively, and 1 or 5 for the remaining tissues. To remove contaminating genomic DNA, total RNA was treated with DNase I. About 500 ng of total RNA of each sample was reverse transcribed with random 6mers using Primescript™ RT reagent kit (Takara, Ohtsu, Japan).

Measurement of mRNA levels

Quantitative real-time RT-PCR (qPCR) was used to measure mRNA levels. The qPCR was performed by Mx3000P™ Real-Time PCR System (Stratagene, La Jolla, CA) using FastStart Universal SYBR Green Master (Roche, Tokyo, Japan) including cDNA reverse transcribed from RNAs extracted from the tissues and SYBER Green with primers for *Gb'per* (*G. bimaculatus period*, GenBank/EMBL/DDBJ accession no. BAG48878), *Gb'tim (G. bimaculatus timeless*, BAJ16356), *Gb'Clk* (*G. bimaculatus Clock*, BAM76759), *Gb'cyc* (*G. bimaculatus cycle*, BAN28450), and *Gb'rpl18a* (*G. bimaculatus rpl18a*, DC448653) (Table 5-1; Chapter 3). The results were analyzed with the software associated with the instrument using generated calibration curves with known amounts of RNA. The values were normalized with those of *Gb'rpl18a* at each time point. Results of three to eight independent experiments were used to calculate the mean ± SEM.

Surgical operation

Animals were collected from the colony chamber between 10:00 (Zeitgeber time (ZT) 4; ZT0 corresponds to light-on and ZT12 corresponds to light-off) and 14:00 (ZT8) for operation. The outer two neuropils of the optic

lobe, the lamina and medulla (hereafter referred to as the ''optic lobe''), which are the locus of the central circadian pacemaker in this cricket (Tomioka and Chiba, 1984, 1992), were surgically removed under CO_2 anesthesia. An incision was made along the margin of the compound eye, the eye capsule was pried open, and the optic stalk and optic nerves were cut with a pair of fine scissors to remove the optic lobe. The eye capsule was then placed back to the original position. The whole procedure took about 5 min.

Statistics

Significance of the daily and circadian rhythmicity in mRNA expression was examined by the single cosinor method (Nelson et al., 1979), fitting a cosine curve of 24 h period using Time Series Analysis Serial Cosinor 6.3 (Expert Soft Technologie Inc.). Significance of difference between values at each time point was examined by Student's *t*-test.

5.4. Results

Expression profile of clock genes in nymphal optic lobes

Expression profiles of clock genes, *Gb'per*, *Gb'tim*, *Gb'cyc,* and *Gb'Clk* were investigated in the nymphal optic lobe under LD12:12 by qPCR. *Gb'per* and *Gb'tim* showed a daily rhythm of mRNA expression with a peak during the late day to early night under LD (cosinor, $P < 0.05$). Their rhythmic profiles were basically similar to those of adult crickets previously reported by Moriyama et al., (2008) and Danbara et al., (2010), with similar amplitudes (peak to trough ratio) of about 2.1- and 2.9-fold for *Gb'per* and *Gb'tim*, respectively (Table 5-2). However, the mRNA levels of *Gb'per* and *Gb'tim* were both about 0.6 times of those of adult tissues and the *Gb'per* mRNA level was significantly lower than that of adult ($P < 0.01$, *t*-test). No significant daily fluctuation was observed in *Gb'cyc* and *Gb'Clk* (Fig. 5-1; cosinor, $P > 0.05$),

although the expression of *Gb'cyc* was rhythmic in adult crickets (Chapter 3). Their mRNA levels were almost similar to those of adult optic lobes.

Expression profiles of clock genes in nymphal extra-optic lobe tissues

It has been previously shown that some extra-optic lobe tissues show rhythmic expression of *Gb'per* and *Gb'tim* in adult crickets (Chapter 4). To examine whether nymphal extra-optic lobe tissues possess this rhythm, expression profiles of clock genes, *Gb'per*, *Gb'tim, Gb'cyc,* and *Gb'Clk* were first investigated in the nymphal nervous tissues, brain and terminal abdominal ganglion (TAG), under LD12:12 and compared them with those in adult tissues. *Gb'per* and *Gb'tim* showed a daily rhythm of mRNA expression under LD in both the brain and TAG (Fig. 5-2; cosinor, $P < 0.01$ for brain and $P < 0.05$ for TAG). Their rhythmic profiles were basically similar to those of adult crickets (Chapter 4) with a peak at early night, except for *Gb'per* in the nymphal TAG, which increased from midday to early night (Fig. 5-2Ba). The amplitudes of their rhythms were not significantly different between nymphs and adults. However, their mRNA levels in the nymphal tissues were significantly lower than those in the adult brain at most time points (Fig. 5-2); the nymphal peak levels were only 0.5 and 0.3 times of adult for *Gb'per* and *Gb'tim*, respectively, in the brain, and 0.8 and 0.4 in the TAG.

No clear rhythm was found in *Gb'cyc* and *Gb'Clk* in either nymphal or adult tissues (Fig. 5-2; cosinor, $P > 0.05$), except for *Gb'cyc* in adult brains, where it was rhythmically expressed with a peak during early night (Fig. 5-2Ac). The highest mRNA levels of *Gb'cyc* and *Gb'Clk* in nymphs were only 0.4 and 0.7 times of those of adults in the brain and 0.5 and 0.6 times in the TAG (Table 5-2), respectively, and the levels were significantly lower than those in the adult tissues at most time points (Fig. 5-2).

The expression of *Gb'per* and *Gb'tim* was the examined in the brain in the second day of constant darkness (DD) to investigate whether the rhythmic

expression was endogenous. As shown in Figure 5-4Aa, *Gb'per* showed a basically similar pattern to that in LD but its level was reduced and no significant rhythm was detected by the cosinor method ($P > 0.05$). The mRNA level of *Gb'tim* was significantly increased and again no significant rhythm was observed (cosinor, $P > 0.05$; Fig. 5-4Ab).

Expression patterns of the clock genes were then examined in two digestive tissues, anterior stomach and mid-gut. In the anterior stomach, no clear rhythmic expression of *Gb'per* and *Gb'tim* was observed in nymphs (cosinor, $P > 0.1$, Fig. 5-3Aa, b), which contrasts to their clear rhythmic expression in adults. The nymphal *Gb'per* mRNA levels were significantly lower at most time points ($P < 0.01$, *t*-test) and the peak mRNA level was only 0.6 times of that of adult (Fig. 5-3Aa), while no significant differences were observed in *Gb'tim* mRNA levels (Fig. 5-3Ab). No significant rhythm was observed in *Gb'cyc* and *Gb'Clk* mRNA levels in both nymphs and adults although some fluctuation was observed (Fig. 5-3Ac, d). In the mid-gut, the nymphal expression patterns of the clock genes were basically similar to those in adults (Fig. 5-3B): a significant daily rhythm of *Gb'tim* expression was detected and the amplitude was about 1.5-fold (cosinor, $P < 0.05$). *Gb'per*, *Gb'cyc* and *Gb'Clk* mRNA showed no clear rhythm (cosinor, $P > 0.05$, Fig. 5-3Ba, c, d), however. The mRNA levels were significantly lower than in adults at most time points except *Gb'Clk*: The highest mRNA levels of *Gb'per*, *Gb'tim* and *Gb'cyc* in nymphs were only 0.6, 0.3 and 0.5 times of those of adults, respectively (Table 5-2; $P < 0.01$, *t*-test).

In the second day of DD, nymphal mid-gut showed a significant reduction of mRNA levels of *Gb'per* and *Gb'tim* (Fig. 5-4B). No significant rhythm was detected in expression of both *Gb'per* and *Gb'tim* (cosinor, $P > 0.05$, Fig. 5-4B), although *Gb'tim* maintained a pattern similar to that in LD with a peak in the subjective night (Fig. 5-4Bb).

Effects of optic lobe removal on mRNA rhythms of nymphal extra-optic lobe tissues

To examine whether the mRNA expression in the extra-optic lobe tissues were affected by the central clock in the optic lobe, mRNA levels of clock genes, *Gb'per* and *Gb'tim* were measured by qPCR in the brain and mid-gut of the nymphal male crickets whose optic lobes were bilaterally removed. The tissues were sampled on the third day after the optic lobe removal. In both brain and mid-gut, neither *Gb'per* nor *Gb'tim* showed a rhythm in their mRNA expression (Fig. 5-5, cosinor, $P > 0.05$). In the mid-gut both genes were downregulated and the reduction of *Gb'tim* was significant (Fig. 5-5B). In the brain, however, *Gb'per* was significantly downregulated but *Gb'tim* was upregulated on optic lobe removal (Fig. 5-5A).

5.5. Discussion

In the cricket *G. bimaculatus,* nymphs show diurnal locomotor activity rhythms, while adults are nocturnally active (Tomioka and Chiba, 1982). The present study revealed that the clock genes *Gb'per* and *Gb'tim* are rhythmically expressed in the optic lobe of nymphal male crickets and that their rhythmic profiles are basically similar to those in the adult optic lobe with a peak at mid night (Moriyama et al., 2008, 2012; Danbara et al., 2010; Fig. 5-1). The common phasing of the *per* and *tim* transcripts rhythm between nymphs and adults is consistent with the fact that electrical activity of the optic lobe and the light induced responses of optic lobe interneurons are always increasing nocturnally, both in nymphs and adults of the cricket *G. bimaculatus* (Tomioka and Chiba, 1992; Uemura and Tomioka, 2006). These facts suggest that the adult and nymphal locomotor rhythms are controlled by the optic lobe circadian clock with a common oscillatory property, but through separate output pathways. This view is consistent with the finding that at the switching phase from a

diurnal to a nocturnal rhythm, both diurnal and nocturnal components are co-expressed (Tomioka and Chiba, 1982).

In contrast to the rhythmic expression of *Gb'per* and *Gb'tim*, *Gb'cyc* was rather constitutively expressed in nymphal optic lobe and the mRNA levels stayed nearly at the basal level of the adult optic lobe. This at least partially explains the lower level of *Gb'per* and *Gb'tim* expression, as *cyc* may be their transcriptional activator (Rutila et al., 1998; Chapter 3). The fact also suggests that the nymphal regulation of *Gb'cyc* transcription may be different from that in adult.

The present study showed that some extra-optic lobe tissues, such as the brain and TAG, exhibit an oscillation even in a nymphal stage under LD (Fig. 5-2). The rhythmic profiles were basically similar to those of the adult tissues (Chapter 4), while the expression levels of the clock genes were considerably lower than those of adult. In the TAG and mid-gut, only *Gb'tim* was rhythmically expressed in LD in nymphs but with reduced mRNA levels (Fig. 5-3B). Basically similar expression patterns were reproduced for *Gb'per* and *Gb'tim* in the brain and for *Gb'tim* in the mid-gut in DD, although the rhythms were not statistically significant (Fig. 5-4). This suggests that the extra-optic lobe oscillations may be so weak they are that rapidly damped out in DD conditions, or their self-sustainability may have not been established at this point in development. The lower level of mRNA expression and weak rhythmicity may be partially attributable to the lower and arrhythmic expression of *Gb'cyc* and *Gb'Clk* in nymphal tissues, since they are most probable transcriptional activator for *per* and *tim* (Allada et al., 1998; Rutila et al., 1998; Moriyama et al., 2012; Chapter 3).

Interestingly, expression levels of the clock genes were enhanced in the extra-optic lobe tissues and the oscillations occurred after adult emergence. In *Drosophila*, the activation of clock genes (*per* and *tim*) occurred at different metamorphic stages in a tissue-specific manner (Giebultowicz et al., 2001). Such

tissue-by-tissue activation of different clock genes suggests that oscillations of peripheral tissues may be turned on by tissue-specific signals or tissue-specific levels of a common signal.

The enhancement of the gene expression and the rhythm in the extra-optic lobe tissues may have significance for their physiological function. In the nymphal tissues, the daily rhythms as well as the level of the mRNA expression were weak. This likely shows that daily rhythms may not be important for tissue function. After the imaginal molt, the daily behavioral rhythm becomes more robust and day-night difference of activity becomes distinct (Tomioka and Chiba, 1982). This probably leads to a clearer daily feeding rhythm and forms a daily rhythm in the digestive system including mid-gut and anterior stomach.

The mRNA expression rhythm of *Gb'per* and *Gb'tim* persisted even after the optic lobe removal in the brain and mid-gut of adult crickets (Chapter 4). Similar circadian oscillations independent of the central clock have been reported in insects. In *Drosophila*, for example, many organs show circadian rhythms that persist in an isolated and cultured condition (Hege et al., 1997; Plautz et al., 1997). In moths, clock gene expression rhythms persist even after the central clock is removed (Merlin et al., 2007, 2009). However, the mRNA expression rhythm disappeared after optic lobe removal in tissues outside the optic lobe in nymphal crickets (Fig. 5-5), suggesting that these rhythms are highly dependent on the optic lobe pacemaker. Similarly, in the cockroach, *Leucophaea maderae*, the rhythms of odor sensitivity of the antenna and light sensitivity of the compound eyes are driven by the central clock (Page and Koelling, 2003; Wills et al., 1985). It remains to be elucidated why the antennae and compound eyes are still highly dependent on the central pacemaker even in adults, while many adult tissues possess a more central-independent oscillation.

How the central clock influences the extra-optic lobe oscillations in

nymphs remains to be answered. There may be two pathways for this control, i.e. neural and humoral. For example, in *Drosophila*, the clock in the prothoracic gland receives a control from the central clock neurons: an ablation of cerebral clock neurons expressing a neuropeptide, pigment dispersing factor (PDF), or null mutation of *pdf* significantly affects the molecular rhythm of prothoracic gland and the resultant eclosion rhythm (Myers et al., 2003). In the linden bug, *Pyrrhocoris apterus*, juvenile hormone (JH) acts in the gut to regulate expression of clock related genes *Par domain protein1* and mammalian-type *cryptochrome* (*cry2*) through its receptor methoprene-tolerant together with CLK, and CYC (Bajgar et al., 2013).

Another important question to be answered is by what mechanism the extra-optic lobe oscillations are strengthened at the imaginal molt. The cricket *G. bimaculatus,* shows a diurnal rhythm with a low level of activity during the nymphal stage. After imaginal molt, the cricket becomes nocturnally active with an increase of activity level (Tomioka and Chiba, 1982). The change is tightly coupled to hormonally controlled adult emergence, as extra-larval molting induced by corpora allata transplantation prevents the change (Tomioka et al., 1993). Thus it may be that the enhancement of the extra-optic lobe oscillation is also caused by some hormonal factor associated with adult emergence. The most likely candidate factor is ecdysone since it is known to affect the expression of circadian clock genes such as *vrille* and *Earlygene at 23* in *Drosophila* (Gauhar et al., 2009; Itoh et al., 2011). Identification of the hormonal factor would lead to elucidation of the mechanism underlying the enhancement of the extra-optic lobe oscillation associated with adult emergence.

References

Allada, R., White, N.E., So, W.V., Hall, J.C., Rosbash, M. (1998) A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*. Cell 93: 791-804

Balkenohl, M., Weber, F. (1981) Sind auch bei holometabolen Insekten circadiane Schrittmacher der Aktivitat in den optischen Ganglion

lokalisiert? Mitt Dtsch Ges Allg Angew Entomol 3: 223–227

Bajgar, A., Jindra, M., Dolezel, D. (2013) Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormo ne signaling. Proc Nal Acad Sci USA 110: 4416-4421

Danbara, Y., Sakamoto, T., Uryu, O., Tomioka, K. (2010) RNA interference of *timeless* gene does not disrupt circadian locomotor rhythms in the cricket *Gryllus bimaculatus*. J Insect Physiol 56: 1738-1745

Gauhar, Z., Sun, L.V., Hua, S., Mason, C.E., Fuchs, F., Li, T.R., Boutros, M., White, K.P. (2009) Genomic mapping of binding regions for the Ecdysone receptor protein complex. Genome Res 19**:** 1006-1013

Giebultowicz, J.M., Invanchenko, M., Vollintine, T. (2001) Organization of the insect circadian system: spatial and developmental expression of clock genes in peripheral tissues of *Drosophila melanogaster*. In: Denlinger DL, Giebultowicz JM, Saunders DS (eds) Insect timing: circadian rhythmicity to seasonality. Elsevier Science B. V, Amsterdam: pp 31–42

Handler, A.M., Konopka, R.J. (1979) Transplantation of a circadian pacemaker in *Drosophila*. Nature 279: 236–238

Hardin, P.E. (2006) Essential and expendable features of the circadian timekeeping mechanism. Curr Opin Neurobiol 16: 686–692

Hege, D.M., Stanewsky, R., Hall, J.C. and Giebultowicz, J.M. (1997) Rhythmic expression of a PER-reporter in the malpighian tubules of decapitated *Drosophila*: evidence for a brain-independent circadian clock. J Biol Rhythms 12: 300-308

Ito, C., Goto, S.G., Shiga, S., Tomioka, K., Numata, H. (2008) Peripheral circadian clock for the cuticle deposition rhythm in *Drosophila melanogaster*. Proc Natl Acad Sci U S A 105: 8446–8451

Itoh, T.Q., Tanimura, T., Matsumoto, A. (2011) Membrane-bound transporter controls the circadian transcription of clock genes in *Drosophila*. Genes Cells 16:1159-1167

Kasai, M., Chiba, Y. (1987) Effects of optic lobe ablation on circadian activity in the mosquito, *Culex pipiens pallens*. Physiol Entomol 12: 59–65

Merlin, C., Lucas, P., Rochat, D., Francois, M.C., Maı¨be`che-Coisne´, M., Jacquin-Joly, E. (2007) An antennal circadian clock and circadian rhythms in peripheral pheromone reception in the moth Spodoptera littoralis. J Biol Rhythms 22: 502–514

Merlin, C., Gegear, R.J., Reppert, S.M. (2009) Antennal circadian clocks coordinate sun compass orientation in migratory monarch butterflies. Science 325: 1700–1704

Moriyama, Y., Sakamoto, T., Karpova, S.G., Matsumoto, A., Noji, S., Tomioka, K. (2008) RNA interference of the clock gene period disrupts circadian rhythms in the cricket *Gryllus bimaculatus*. J Biol Rhythms 23:

308–318
Moriyama, Y., Kamae, Y., Uryu, O., Tomioka, K. (2012) *Gb'Clock* Is Expressed in the Optic Lobe and Required for the Circadian Clock in the Cricket *Gryllus bimaculatus*. J Biol Rhythms 27: 467-477
Myers, E.M., Yu, J. and Sehgal, A. (2003) Circadian control of eclosion: interaction between a central and peripheral clock in *Drosophila melanogaster*. Curr Biol 13: 526-533
Nelson, W., Tong, Y., Lee, J., Halberg, F. (1979) Methods for cosinor-rhythmometry. Chronobiologia 6: 305–323
Page, T.L. (1982) Transplantation of the cockroach circadian pacemaker. Science 216: 73–75
Page, T.L., Koelling, E. (2003) Circadian rhythm in olfactory response in the antennae controlled by the optic lobe in the cockroach. J Insect Physiol 49: 697–707
Pittendrigh, C.S. (1974) Circadian oscillations in cells and the circadian organization of multicellular systems. In Schmitt FO, Worden GO (eds) The Neurosciences: Third Study Program. MIT Press, Cambridge, Massachusetts, pp 437-458
Plautz, J.D., Kaneko, M., Hall, J.C., Kay, S.A. (1997) Independent photoreceptive circadian clocks throughout *Drosophila*. Science 278: 1632–1635
Rutila, J.E., Suri, V., Le, M., So, W.V., Rosbash, M. and Hall, J.C. (1998) CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. Cell 93: 805-814
Stanewsky, R. (2002) Clock mechanisms in *Drosophila*. Cell Tissue Res 309: 11–26
Tomioka, K., Chiba, Y. (1982) Post-embryonic development of circadian rhythm in the cricket, *Gryllus bimaculatus*. J Comp Physiol A 147: 299–304
Tomioka, K., Chiba, Y. (1984) Effects of nymphal stage optic nerve severance or optic lobe removal on the circadian locomotor rhythm of the cricket, *Gryllus bimaculatus*. Zoolog Sci 1: 385–394
Tomioka, K., Chiba, Y. (1992) Characterization of optic lobe circadian pacemaker by *in situ* and *in vitro* recording of neuronal activity in the cricket *Gryllus bimaculatus*. J Comp Physiol A 171: 1–7
Tomioka, K., Seto, N., Okada, S-I., Terada, T., Chiba, Y. (1993) Analysis of postembryonic development of locomotor activity rhythm by corpora allata inplantation in the cricket *Gryllus bimaculatus*. Comp Biochem Physiol 106A: 43-48
Tomioka, K., Matsumoto, A. (2010) A comparative view of insect circadian clock systems. Cell Mol Life Sci 67: 1397-1406
Truman, J.W. (1974) Physiology of insect rhythms. IV. Role of the brain in the regulation of the flight rhythm of the giant silkmoths. J Comp

Physiol A 95: 281–296

Uemura, H., Tomioka, K. (2006) Postembryonic changes in circadian photo-responsiveness rhythms of optic lobe interneurons in the cricket *Gryllus bimaculatus*. J Biol Rhythms 21: 279-289

Uryu, O., Tomioka, K. (2010) Circadian oscillations outside the optic lobe in the cricket *Gryllus bimaculatus*. J Insect Physiol 56: 1284-1290

Uryu, O., Karpova, S.G., Tomioka, K. (2013) The clock gene cycle plays an important role in the circadian clock of the cricket *Gryllus bimaculatus*. J Insect Physiol 59: 697-704

Vitaterna, M.H., Selby, C.P., Todo, T., Niwa, H., Thompson, C., Fruechte, E.M., Hitomi, K., Thresher, R.J., Ishikawa, T., Miyazaki, J., Takahashi, J.S., Sancar, A. (1999) Differential regulation of mammalian period genes and circadian rhyth micity by cryptochromes 1 and 2. Proc Natl Acad Sci U S A 96: 12114-12119.

Weitzel, G., Rensing, L. (1981) Evidence for cellular circadian rhythms in isolated fluorescent dye-labelled salivary glands of wild type and an arrhythmic mutant of *Drosophila melanogaster*. J Comp Physiol B 143: 229-235

Wills, S.A., Page, T.L., Colwell, C.S. (1985) Circadian rhythms in the electroretinogram of the cockroach. J Biol Rhythms 1: 25–37

Yuan, Q., Metterville, D., Briscoe, A.D., Reppert, S.M. (2007) Insect cryptochromes: gene duplication and loss define diverse ways to construct insect circadian clocks. Mol Biol Evol 24: 948–955

Table 5-1. Primers used for quantitative RT-PCR.

Genes	Forward	Reverse
period	5′-AAGCAAGCAAGCATCCTCAT-3′	5′-CTGAGAAAGGAGGCCACAAG-3′
timeless	5′- GACGAATGCATTTGGTTGTG -3′	5′- CATCCGTTGACCCTATTTGC -3′
cycle	5′-GGCCGAAGCTCATAAAGTGG-3′	5′-AACCGCACAAAGGAACCATC-3′
Clock	5′-AATGACCGTAGTCGAGAAAGTGAAG-3′	5′-TTGCGATGATTGAGGTTGTTG-3′
rpl18a	5′-GCTCCGGATTACATCGTTGC-3′	5′-GCCAAATGCCGAAGTTCTTG-3′

Table 5-2. Summary of the expression profile of clock genes in the optic lobe (OL), brain, terminal abdominal ganglion (TAG), anterior stomach (A-stomach) and mid-gut.

Tissue	Gene	Rhythm[a]		amplitude[b]		mRNA level[c]	
		Nymph	Adult	Nymph	Adult	Nymph	Adult
OL	*per*	+	+	2.1	1.7	0.33	0.54
	tim	+	+	2.9	2.9	0.057	0.096
	cyc	—	+	—	1.5	1.2	1.5
	Clk	—	—	—	—	0.32	0.37
Brain	*per*	+	+	1.6	1.7	0.2	0.38
	tim	+	+	2.3	2.5	0.057	0.19
	cyc	—	+	—	1.7	0.77	1.9
	Clk	—	—	—	—	0.48	0.65
TAG	*per*	+	+	1.9	1.3	0.34	0.43
	tim	+	+	1.5	1.8	0.069	0.17
	cyc	—	—	—	—	1.9	3.5
	Clk	—	—	—	—	0.41	0.69
A-Stomach	*per*	—	+	—	1.3	0.21	0.38
	tim	—	—	—	—	0.15	0.15
	cyc	—	—	—	—	0.43	0.57
	Clk	—	—	—	—	0.086	0.66
Mid-gut	*per*	—	+	—	1.4	0.12	0.21
	tim	+	+	1.5	1.4	0.033	0.11
	cyc	—	—	—	—	0.84	1.7
	Clk	—	—	—	—	0.081	0.088

a) the single cosinor method was used to detect the rhythm: +, $P < 0.05$; —, $P > 0.05$.

b) amplitude indicates the peak to trough ratio.

c) mRNA level indicates the highest value relative to that of *rpl18a*.

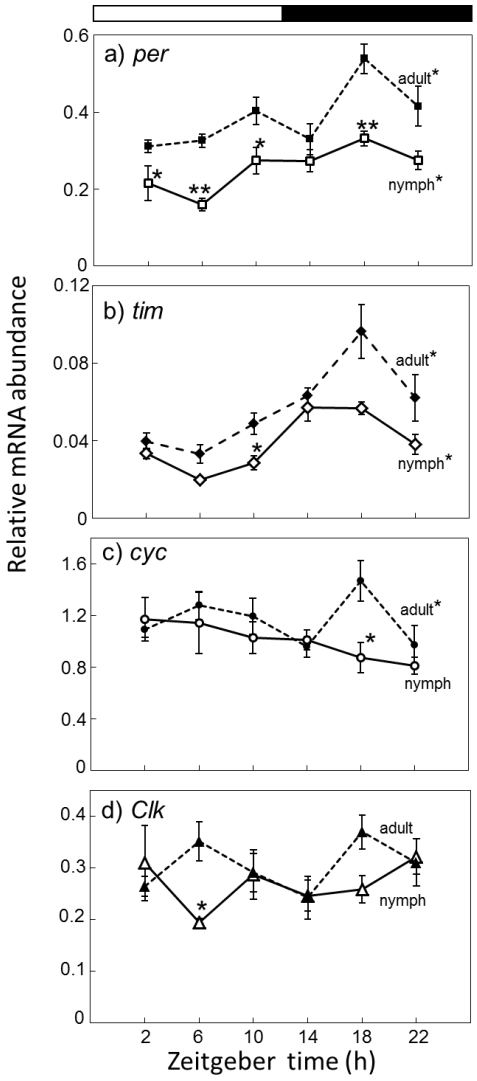

Fig. 5-1. Transcripts abundance of the 4 clock genes (*Gb'per*, *Gb'tim*, *Gb'cyc* and *Gb'Clk*) over time in the cricket optic lobe under LD at 25°C.

Solid and broken lines are for nymphal and adult crickets, respectively. The abundance of mRNAs was measured by quantitative real-time RT-PCR with total RNA extracted from the optic lobe of adult and nymphal crickets that were collected at 4 h intervals starting at 2 h after light-on (ZT2). The values are shown with SEM as relative amount to those of *Gb'rpl18a* mRNA. Black and white bars indicate night and day, respectively. Data for adults were adopted from Chapter 3. **P* < 0.05, ***P* < 0.01, *t*-test, vs adult crickets. Asterisks at the shoulder of nymph and adult indicate significance of the rhythm (cosinor, P < 0.05).

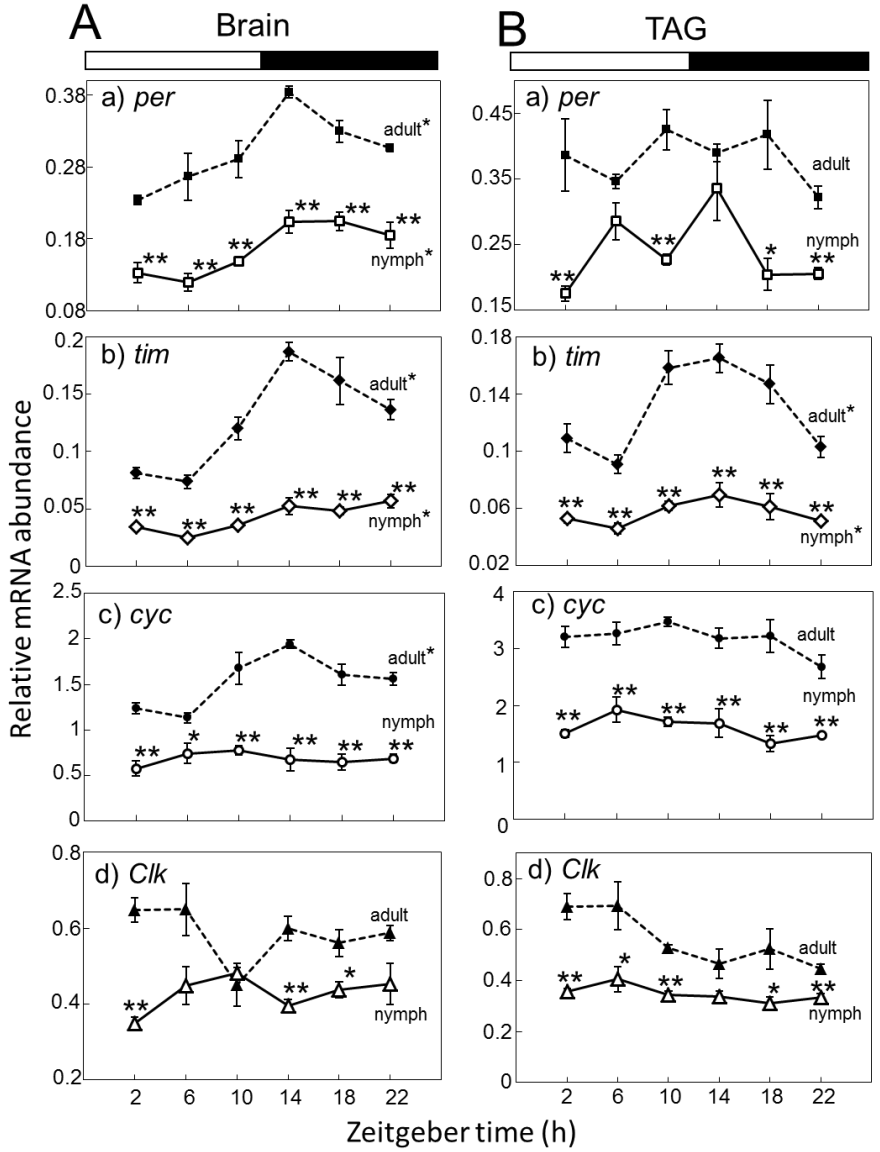

Fig. 5-2. Transcripts abundance of the 4 clock genes (*Gb'per*, *Gb'tim*, *Gb'cyc* and *Gb'Clk*) over time in adult and nymphal brain (A) and terminal abdominal ganglion (TAG) (B), under LD at 25°C.

Solid and broken lines are for nymphal and adult crickets, respectively. The abundance of clock genes mRNA was measured by quantitative real-time RT-PCR with total RNA extracted from the tissues collected at 4h intervals starting at 2h after light-on (ZT2). The values shown are relative to the amount of *Gb'rpl18a* mRNA. Vertical bars indicate SEM. *$P < 0.05$, **$P < 0.01$, *t*-test, vs adult crickets. Asterisks at the shoulder of nymph and adult indicate significance of the rhythm (cosinor, $P < 0.05$). Black and white bars indicate night and day, respectively. For other explanations see text.

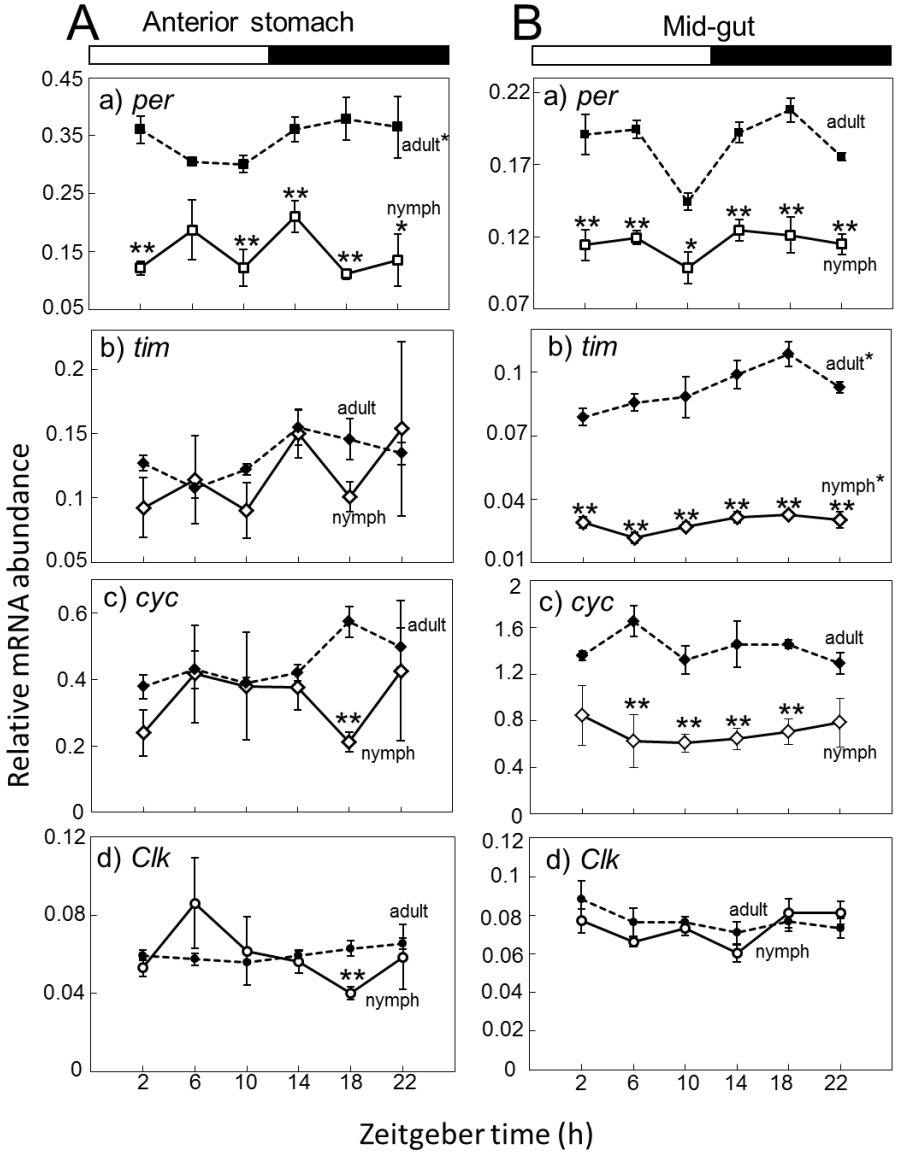

Fig. 5-3. Transcripts abundance of the 4 clock genes (*Gb'per*, *Gb'tim*, *Gb'cyc* and *Gb'Clk*) overtime in adult and nymphal anterior stomach (A) and mid-gut (B), under LD at 25°C.

Solid and broken lines are for nymphal and adult crickets, respectively. The abundance of clock genes mRNA was measured by quantitative real-time RT-PCR with total RNA extracted from the tissues collected at 4 h intervals starting at 2 h after light-on (ZT2). The value shown is relative to the amount of *Gb'rpl18a* mRNA. Vertical bars indicate SEM. **P* < 0.05, ***P* < 0.01, *t*-test, vs adult crickets. Asterisks at the shoulder of nymph and adult indicate significance of the rhythm (cosinor, *P* < 0.05). Black and white bars indicate night and day, respectively. For further explanations see text.

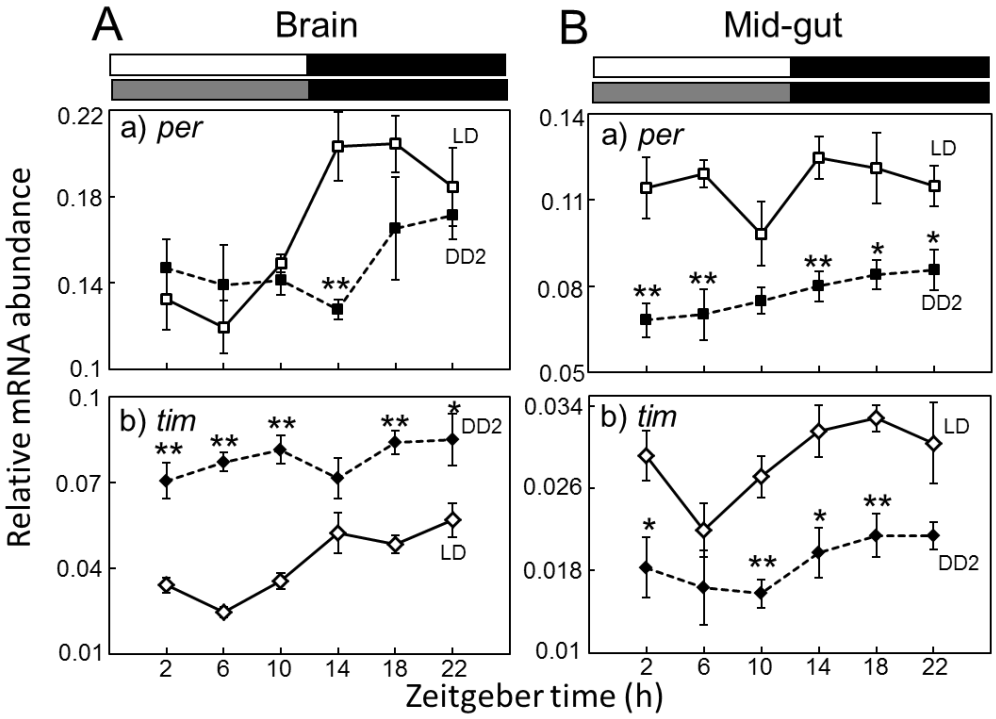

Fig. 5-4. Transcripts abundance of *per* and *tim* over time under LD and DD at 25°C in nymphal brain (A) and mid-gut (B).

Solid and broken lines are for LD and DD conditions, respectively. Data for LD are replotted from Figs. 5-2 and 5-3. For DD total RNA was extracted from the tissues that were collected at 4 h interval starting at 2 h after projected light-on (Circadian time 2) on the second day of DD. White, black and gray bars indicate light phase, night/subjective night and subjective day, respectively. For further explanations see text.

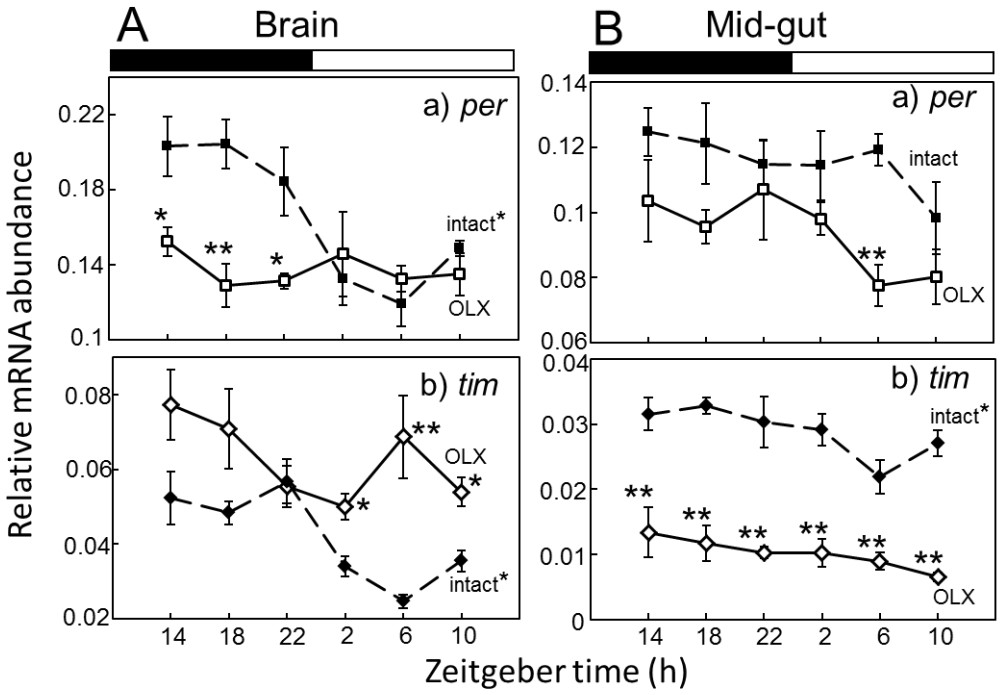

Fig. 5-5. Transcripts abundance of *per* and *tim* over time under LD at 25°C in nymphal brain (A) and mid-gut (B) 48 h after bilateral optic lobe removal. Solid and broken lines are for the crickets receiving the optic lobe removal (OLX) and for intact crickets duplicated from Figs. 5-2 and 5-3. The mRNA abundance was measured by quantitative real-time RT-PCR with total RNA extracted from the tissues of adult and nymphal crickets that were collected at 4 h intervals starting at 2 h after light-off (ZT14). The values shown are relative to the amount of *Gb'rpl18a* mRNA. Vertical bars indicate SEM. **P* < 0.05, ***P* < 0.01, *t*-test, vs intact crickets. Black and white bars indicate night and day, respectively. Note that no clear rhythm was detected in both brain and mid-gut after the optic lobe removal. Asterisks at the shoulder of intact indicate significance of the rhythm (cosinor, *P* < 0.05).

Chapter 6.

General discussion

The goal of this study was expansion of the fundamental knowledge of the clock system in the cricket and establishment of the cricket *Gryllus bimaculatus* as a model suitable for molecular study of the circadian clock system. In chronobiology, forward genetics was the major methodology to dissect the molecular mechanism of circadian clocks in the model animals such as *Drosophila* (Antoch et al., 1997; Konopka and Benzer, 1971). Unfortunately, however, the forward genetic approach could not be adopted in the cricket, because of a lack of genetic information and tools. Hence, in this study, a reverse genetic approach was adopted to analyze the molecular oscillatory mechanism. In this study, the following three issues were addressed: (1) fundamental information for utilizing RNAi technique for effective knock-down of cricket's gene; (2) molecular oscillatory mechanism of the circadian clock in the cricket; (3) relationships between central and peripheral clocks in the circadian organization.

6.1. Advantage of RNAi in non-model insects

In the present study, fundamental information for utilizing RNAi technique in long-lasting experiments was provided. Systemic RNAi is a powerful tool to investigate gene function in non-model insects such as locusts, bees, and beetles (Bucher et al., 2002; Farooqui et al., 2004; He et al., 2006). However, the range of its application was not fully understood in insects. The present study showed that the clock-gene knock-down persisted for a long period in two insect species, *G. bimaculatus* and *T. domestica*. The long-lasting RNAi effect is convenient for chronobiological studies that require monitoring

of physiological functions over long periods of time.

An interesting question is why RNAi is effective in crickets and firebrats. The efficiency of the RNAi would be gene, tissue, or species-dependent. In *Anopheles gambiae* salivary glands, RNAi is ineffective because the expression level of Dicer is too low to cleavage dsRNA into siRNA (Boisson et al., 2006). The salivary glands are also known to have little uptake of siRNA compared with other tissues. The dsRNA uptake is an active process involving receptor-mediated endocytosis and is found even in *Caenorhabditis elegans* (Saleh et al., 2006). Thus, crickets and firebrats might have an evolutionarily conserved dsRNA uptake mechanism.

6.2. Possible evolutional position of the cricket circadian clock

A full length cDNA sequence of the clock gene *Gb'cyc* was obtained through molecular cloning. Structural analysis revealed that several functional domains of the putative *Gb'*CYC protein have high similarity to those of other insect species. It is an interesting point that *Gb'*CYC contains a transactivation domain called BMAL C-terminal region ("BCTR") like its mammalian ortholog, *Bmal1* (Chang and Reppert, 2003), while *Dm'*CYC lacks it. The BCTR was also found in many other insect CYCs including *Ap'*BMAL and *Td'*CYC (Chang et al., 2003; Kamae et al., 2010). Thus it might be acquired at an early evolutionary stage and is likely wide spread in the animal kingdom. In *A. pernyi* and *T. domestica*, their CLK lacks C-terminal glutamine-rich transactivation domain (poly-Q) which is thought to be a transactivation domain in the fruit fly and mammalian CLK. This fact suggests that either CYC or CLK has a role in

transcriptional activation in insects. In the cricket, however, both CYC and CLK have a transactivation domain (Moriyama et al., 2012), making cricket a particularly interesting species in view of circadian clock evolution. This fact also suggests that the glutamine-rich transactivation domains found in *D. melanogaster*, *G. bimaculatus* and mouse CLKs are not conserved from an ancestral CLK protein. The ancestral CLK might lack polyglutamine stretches and acquired them independently in *D. melanogaster*, *G. bimaculatus,* and vertebrate lineages.

The measurement of mRNA levels of *Gb'cyc* in the optic lobe revealed that *Gb'cyc* expression shows a pattern similar to that of the *Gb'per* and *Gb'tim*, with a peak around mid-night under LD. *Gb'Clk* mRNA shows an arrhythmic expression, but *Gb'cyc* mRNA is expressed in a rhythmic manner like in mammals. Therefore, the CLK-CYC heterodimer plays a role as a transcriptional activator but in a manner different from that in *D. melanogaster* (Moriyama et al., 2012). It seems likely that the transcription of *Gb'cyc* is regulated in a way similar to that in mammals, in which *Rev-erb* represses and *Ror* activates the transcription of *Bmal1* (Preitner et al., 2002; Sato et al., 2004; Cho et al., 2012). Actually the orthologs of *Rev-erb* and *Ror* have been cloned in the cricket. They are the nuclear receptor E75 and HR3. *Gb'per* is required for the sufficient expression of *Gb'cyc* at least in LD (Chapter 3). Since *Gb'per* is also required for *Gb'Clk* (Moriyama et al., 2012), it probably plays a common role in regulation of these two genes. Interestingly, *Gb'Clk* oscillated in *cyc* RNAi crickets. It should be worth examining whether the mechanisms for rhythmic expression of *cyc* and *Clk* coexist in the cricket (Fig. 6-1). Taken together, the

circadian clock in *G. bimaculatus* has a unique molecular oscillatory mechanism with features of both *Drosophila* and mammalian clocks.

6.3. The central and peripheral circadian clock system in the cricket

The present study revealed that some tissues outside the optic lobe in the cricket show rhythmic expression of clock genes, revealing the existence of circadian clock in those tissues. The peripheral clocks are self-sustained oscillators with molecular machinery slightly different from that of the central clock. Some other insects also have the peripheral clocks. For example, the MTs have a complete circadian clock system in *Drosophila,* which can operate and synchronize to LD (Hege et al., 1997; Giebultowicz et al., 2000). In moths and butterflies, the clock gene *per* is rhythmically expressed in the antenna (Merlin et al., 2006; Schuckel et al., 2007). Furthermore, mammalian peripheral organs and tissues also contain clocks (Stratmann and Schibler 2006; Dibner et al., 2010). The rat liver suspension culture showed circadian rhythm, indicating the presence of self-sustaining clock in the periphery (Langner et al., 1972). Thus, the evolutionally conserved peripheral clock system should have great importance in animals.

The central and peripheral relationships are different among species. In *Drosophila*, many organs such as legs, antennae, wings and MTs show circadian rhythms that maintain oscillations in an isolated and cultured condition (Hege et al., 1997; Plautz et al., 1997). These tissues can be entrained to light cycles and temperature cycles *in vitro* (Levine et al., 2002; Glaser and Stanewsky 2005). The expression of the *Clk* gene shows a strong circadian oscillation in peripheral

organs *in vivo* and in culture in zebrafish, showing that endogenous oscillators exist in peripheral organs (Whitmore et al., 1998). The zebrafish peripheral circadian oscillator is also directly light entrained (Whitmore et al., 2000). Thus, these peripheral clocks can oscillate independently of the central clock. On the other hand, the peripheral clocks of other species are apparently dependent on the central clock. In cockroaches, the antennal odor sensitivity rhythm measured by electroantennogram (EAG) is driven by the central clock, since it is lost when the optic tracts are bilaterally severed (Page and Koelling, 2003). The mammalian peripheral clocks have no direct entrainment mechanism to environmental cycles. Given the hierarchical architecture of the circadian timing system, the peripheral clocks may be coordinated by systemic cues emanating from the central clock (Dibner et al., 2010). The present study showed that the dependency of peripheral clocks on the central clock has been examined at the molecular level in the cricket. The tissues outside the optic lobe such as the brain, terminal abdominal ganglion (TAG), anterior stomach and mid-gut exhibited rhythmic expression of clock genes *per* and *tim* mRNAs. However, the amplitude of the mRNA rhythms varied among those rhythmic tissues. Removal of the optic lobe, the central clock tissue, differentially affected the rhythms. These facts suggest that rhythms outside the optic lobe receive control from the optic lobe to different degrees, and more interestingly that the oscillatory mechanism may be different from that of the central clock (Fig. 6-2). Taken these data together, it seems rather general that the central clock more or less regulates the peripheral clocks even when they can oscillate independently.

For photic entrainment of the cricket's peripheral tissues, the light input

may be mediated by the optic lobe central clock, like in mammalian peripheral clocks of which phase is set by the central clock in the suprachiasmatic nuclei through some signals (Dibner et al., 2010; Kalsbeek et al., 2011). This statement is based on the fact that the compound eye is the only circadian photoreceptor (Tomioka and Chiba, 1984) and that cricket's peripheral clocks are influenced by the presence of the central clock (Chapter 4). However, in *Drosophila* all peripheral tissues could obtain light input directly via CRY because fly's cuticle can easily transmit light, but it is still elusive in some tissues (Ivanchenko et al., 2001). It is an important question why the peripheral clocks have either indirect or direct pathway for photic entrainment in a species dependent manner.

6.4. Coordination of the central and peripheral circadian clock system in the cricket

The present study showed that the mRNA expressions of the clock genes are dependent on the optic lobe clock in some extra-optic lobe tissues. The pathway through which the central clock influences the extra-optic lobe oscillations is to be elucidated. In *Drosophila*, the clock in the prothoracic gland receives a control from the central clock neurons: an ablation of cerebral clock neurons expressing a neuropeptide, pigment dispersing factor (PDF), or null mutation of *pdf* significantly affects the molecular rhythm of prothoracic gland and the resultant eclosion rhythm (Myers et al., 2003). The PDF is also widely distributed in the cricket optic lobes and brain (Singaravel et al., 2003). In the linden bug, *Pyrrhocoris apterus*, juvenile hormone (JH) acts in the gut to regulate expression of clock related genes through its receptor (Bajgar et al., 2013). Removal of the optic lobe, the central clock tissue, differentially affected the

rhythms: the anterior stomach lost the rhythm of both *per* and *tim*; in the midgut and TAG, *tim* expression became arrhythmic but *per* maintained its rhythmic expression; a persistent rhythm with a shifted phase was observed for both *per* and *tim* mRNA rhythms in the brain (Chapter 4). These data suggest that rhythms outside the optic lobe receive control from the optic lobe to different degrees through neural and humoral signals.

Furthermore, the present study showed that among the nymphal extra-optic lobe tissues, the brain and TAG showed a rhythmic expression of *per* and *tim,* the mid-gut only of *tim,* and the anterior-stomach none of them. The mRNA levels of clock genes were significantly lower than those in adults. These results suggest that the rhythms outside the optic lobe are weak in nymphs and may become robust after the imaginal molt (Chapter 5). This fact may be related to characteristic behavior of the cricket, *G. bimaculatus*. In the cricket *G. bimaculatus,* nymphs show a diurnal rhythm, while adults, on the contrary, are nocturnal. This rhythm reversal occurred after the imaginal molt (Tomioka and Chiba 1982). The circadian clock in the optic lobe has been shown to have a common physiological property in nymphs and adults (Tomioka and Chiba, 1992), suggesting that changes outside the optic lobe are related to the phase reversal in overt activity rhythms.

The cricket *G. bimaculatus,* shows a diurnal rhythm with a low level of activity during the nymphal stage and becomes nocturnally active with an increase of activity level after imaginal molt (Tomioka and Chiba, 1982). The change is tightly coupled to hormonally controlled adult emergence because extra-larval molting induced by corpora allata transplantation prevented the

change (Tomioka et al., 1993). The present study showed that the phases of the clock gene transcripts rhythms are common between nymphs and adults in the optic lobe and extra-optic lobe tissues (Chater 5). It is consistent with the fact that electrical activity of the optic lobe and the light induced responses of optic lobe interneurons are always nocturnally increasing both in nymphs and adults of the cricket *G. bimaculatus* (Tomioka and Chiba, 1992; Uemura and Tomioka, 2006). It is thus likely that the adult and nymphal locomotor rhythms are controlled by the optic lobe circadian clock with a common oscillatory property but through separate output pathways. It may be plausible that the enhancement of the extra-optic lobe oscillation is also caused by some hormonal factor associated with adult emergence. The most likely candidate factor is ecdysone since it is known to affect the expression of circadian clock genes such as *vrille* and *Earlygene at 23* in *Drosophila* (Gauhar et al., 2009; Itoh et al., 2011). Then, the circadian clocks in peripheral tissues may control physiological function through output pathways. The fruit fly shows a circadian rhythm of feeding which is partially controlled by circadian clocks in peripheral tissues including fat bodies (Xu et al., 2008). In insects the fat body has an important role in energy metabolism like the liver in mammals (Scott et al., 2004). Taken together, the rhythm reversal may depend on changes in the output pathway not in the oscillator (Fig. 6-3).

6.5. Conclusion

The cricket *Gryllus bimaculatus* now becomes a good model for molecular study of the circadian clock system, because the present study revealed the following three points: (1) RNAi is effective for knocking-down cricket's clock genes; (2) molecular mechanism of the circadian clock in the cricket has a unique feature; (3) the rhythms outside the optic lobe are dependent on the optic lobe clock in a tissue dependent manner and may become robust after the imaginal molt. The present study has partially revealed the molecular oscillatory mechanism of the circadian clock and the central and peripheral relationships of the circadian organization in the cricket *G. bimaculatus*. The results would contribute toward understanding the insect clock mechanism. Among non-model insects the cricket is the one in which molecular study of the circadian clock system is most advanced. Furthermore, the cricket has a unique feature that the rhythm reversal occurs after the imaginal molt. The cricket is suitable model insect for addressing the issue how diurnal and nocturnal rhythms are controlled by a single oscillator.

Several important issues remain to be addressed on the mechanism of circadian clock system in the cricket *G. bimaculatus*. The circadian pacemaker neurons are yet to be elucidated. To identify the clock cells, immunohistochemistry or *in situ* hybridization may be suitable method. Once the cells are identified, time course of expression and subcellular localization of the clock gene product proteins could be examined. Through these studies the cricket *G. bimaculatus* will become a unique model insect for molecular study of the circadian clock system.

References

Bajgar, A., Jindra, M., Dolezel, D. (2013) Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormon e signaling. Proc Nal Acad Sci USA 110: 4416-4421

Boisson, B., Jacques, J.C., Choumet, V., Martin, E., Xu, J., Vernick, K., Bourgouin, C. (2006) Gene silencing in mosquito salivary glands by RNAi. FEBS Lett 580:1988-92.

Bucher, G., Scholten, J., Klingler, M. (2002) Parental RNAi in *Tribolium* (Coleoptera). Curr Biol 12: 85-86.

Chang, D.C., McWatters, H.G., Williams, J.A., Gotter, A.L., Levine, J.D., Reppert, S.M. (2003) Constructing a feedback loop with circadian clock molecules from the silkmoth, *Antheraea pernyi*. J Biol Chemistry 278:38149.

Chang, D.C., Reppert, S.M. (2003) A novel C-terminal domain of *Drosophila* PERIOD inhibits dCLOCK:CYCLE-mediated transcription. Curr Biol 13:758-762.

Cho, H., Zhao, X., Hatori, M., Yu, R.T., Barish, G.D., Lam, M.T., Chong, L.W., DiTacchio, L., Atkins, A.R., Glass, C.K., Liddle, C., Auwerx, J., Downes, M., Panda, S., Evans, R.M. (2012) Regulation of circadian behavior and metabolism by REV-ERB-α and REV-ERB-β. Nature 485:123-127.

Danbara, Y., Sakamoto, T., Uryu, O., Tomioka, K. (2010) RNA interference of *timeless* gene does not disrupt circadian locomotor rhythms in the cricket *Gryllus bimaculatus*. J Insect Physiol 56:1738-1745.

Dibner, C., Schibler, U., Albrecht, U. (2010) The mammalian circadian timing system: organization and coordination of central and peripheral clocks. Ann Rev Physiol 72:517–549

Farooqui, T., Vaessin, H., Smith, B.H. (2004) Octopamine receptors in the honeybee (*Apis mellifera*) brain and their disruption by RNA-mediated interference. J Insect Physiol. 50:701-713.

Gauhar, Z., Sun, L.V., Hua, S., Mason, C.E., Fuchs, F., Li, T.R., Boutros, M., White, K.P. (2009) Genomic mapping of binding regions for the Ecdysone receptor protein complex. Genome Res 19: 1006-1013

Glaser, F.T., Stanewsky, R. (2005) Temperature synchronization of the *Drosophila* circadian clock. Curr Biol 15:1352–1363

Giebultowicz, J.W., Stanewsky, R., Hall, J.C., Hege, D.M. (2000) Transplanted *Drosophila* excretory tubules maintain circadian clock cycling out of phase with the host. Curr Biol 10:107–110

He, Z.B., Cao, Y.Q., Yin, Y.P., Wang, Z.K., Chen, B., Peng, G.X., Xia, Y.X.(2006) Role of hunchback in segment patterning of *Locusta migratoria manilensis* revealed by parental RNAi. Dev Growth Differ 48:439-445.

Hege, D.M., Stanewsky, R., Hall, J.C., Giebultowicz, J.M. (1997) Rhythmic expression of a PER-reporter in the malpighian tubules of decapitated *Drosophila*: evidence for a brain-independent circadian clock. J Biol Rhythms 12:300–308.

Ikeno, T., Tanaka, S.I., Numata, H., Goto, S.G. (2010) Photoperiodic diapause under the control of circadian clock genes in an insect. BMC Biology 8: 116.

Itoh, T.Q., Tanimura, T., Matsumoto, A. (2011) Membrane-bound transporter controls the circadian transcription of clock genes in Drosophila. Genes Cells 16:1159-1167

Ivanchenko, M., Stanewsky, R., Giebultowicz, J.M. (2001) Circadian photoreception in *Drosophila*: functions of cryptochrome in peripheral and central clocks. J Biol Rhythms 16:205–215

Kamae, Y., Tanaka, F., Tomioka, K. (2010) Molecular cloning and functional analysis of the clock genes, *Clock* and *cycle*, in the firebrat *Thermobia domestica*. J Insect Physiol 56:1291-1299.

Kalsbeek, A., Yi, C-X., Cailotto, C., la Fleur, S.E., Fliers, E., Buijs, R.M. (2011) Mammalian clock output mechanisms. Essays Biochem 49:137–151

Kotwica, J., Bebas, P., Gvakharia, B.O., Giebultowicz, J.M. (2009) RNA interference of the period gene affects the rhythm of sperm release in moths. Journal of Biological Rhythms 24:25–34.

Langner, R., Rensing, L. (1972) Circadian rhythm of oxygen consumption in rat liver suspension culture: changes of pattern. Z. Naturforsch. Teil B 27:1117–18

Levine, J.D., Funes, P., Dowse, H.B., Hall, J.C. (2002) Advanced analysis of a cryptochrome mutation's effects on the robustness and phase of molecular cycles in isolated peripheral tissues of *Drosophila*. BMC Neurosci 3:5

Merlin, C., Franc¸ois, M.C., Queguiner, I., Maı¨be`che-Coisne´, M., Jacquin-Joly, E. (2006) Evidence for a putative antennal clock in *Mamestra brassicae*:

Molecular cloning and characterization of two clock genes—period and cryptochrome—in antennae. Insect Mol Biol 15:137–145

Myers, E.M., Yu, J., Sehgal, A. (2003) Circadian control of eclosion: interaction between a central and peripheral clock in *Drosophila melanogaster*. Curr Biol 13: 526-533

Nishiitsutsuji-Uwo, J., Pittendrigh, C.S. (1968) Central nervous system control of circadian rhythmicity in the cockroach. II. The pathway of light signals that entrain the rhythm. Zeitschrift feur vergleichende Physiologie 58:1–13

Page, T.L., Koelling, E. (2003) Circadian rhythm in olfactory response in the antennae controlled by the optic lobe in the cockroach. J Insect Physiol 49:697–707

Plautz, J.D., Kaneko, M., Hall, J.C., Kay, S.A. (1997) Independent photoreceptive circadian clocks throughout *Drosophila*. Science 278:1632–1635

Preitner, N., Damiola, F., Molina, L.L., Zakany, J., Duboule, D., Albrecht, U., Schibler, U. (2002) The orphan nuclear receptor REV-ERB alpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110:251-260.

Saleh, M.C., van Rij, R.P., Hekele, A., Gillis, A., Foley, E., O'Farrell, P.H., Andino, R. (2006) The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. Nature Cell Biology 8, 793–802.

Sato, T.K., Panda, S., Miraglia, L.J., Reyes, T.M., Rudic, R.D., McNamara, P., Naik, K.A., FitzGerrald, G.A., Kay, S.A., Hogenesch, J.B. (2004) A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. Neuron 43:527-537.

Schuckel, J., Siwicki, K.K., Stengl, M. (2007) Putative circadian pacemaker cells in the antenna of the hawkmoth *Manduca sexta*. Cell Tissue Res 330:271–278

Stratmann, M., Schibler, U. (2006) Properties, entrainment, and physiological functions of mammalian peripheral oscillators. J Biol Rhythms 21:494–506

Tobback, J., Vuerinckx, K., Boerjan, B., Huybrechts, R. (2012) RNA interference mortality points to noncircadian functions for the clock gene in the desert locust Schistocerca gregaria. Insect Molecular Biology 21: 369–381.

Tomioka, K., Chiba, Y. (1982) Post-embryonic development of circadian

rhythm in the cricket, *Gryllus bimaculatus*. J Comp Physiol A 147: 299– 304

Tomioka, K., Chiba, Y. (1984) Effects of nymphal stage optic nerve severance or optic lobe removal on the circadian locomotor rhythm of the cricket, *Gryllus bimaculatus*. Zool Sci 1:385–394

Tomioka, K., Chiba, Y. (1992) Characterization of optic lobe circadian pacemaker by *in situ* and *in vitro* recording of neuronal activity in the cricket *Gryllus bimaculatus*. J Comp Physiol A 171: 1–7

Tomioka, K., Seto, N., Okada, S-I., Terada, T., Chiba, Y. (1993) Analysis of postembryonic development of locomotor activity rhythm by corpora allata inplantation in the cricket *Gryllus bimaculatus*. Comp Biochem Physiol 106A: 43-48

Uemura, H., Tomioka, K. (2006) Postembryonic changes in circadian photo-responsiveness rhythms of optic lobe interneurons in the cricket *Gryllus bimaculatus*. J Biol Rhythms 21: 279-289

Uryu, O., Tomioka, K. (2010) Circadian oscillations outside the optic lobe in the cricket *Gryllus bimaculatus*. J Insect Physiol 56: 1284-1290

Uryu, O., Karpova, S.G., Tomioka, K. (2013) The clock gene cycle plays an important role in the circadian clock of the cricket *Gryllus bimaculatus*. J Insect Physiol 59: 697-704

Whitmore, D., Foulkes, N.S., Strähle, U., Sassone-Corsi, P. (1998) Zebrafish Clock rhythmic expression reveals independent peripheral circadian pacemakers. Nature Neurosci. 1, 701– 707.

Whitmore, D., Foulkes, N.S., Sassone-Corsi, P. (2000) Light acts directly on organs and cells in culture to set the vertebrate circadian clock. Nature.404:87-91.

Xu, K., Zheng, X., Sehgal, A. (2008) Regulation of feeding and metabolism by neuronal and peripheral clocks in *Drosophila*. Cell Metab 8:289–300

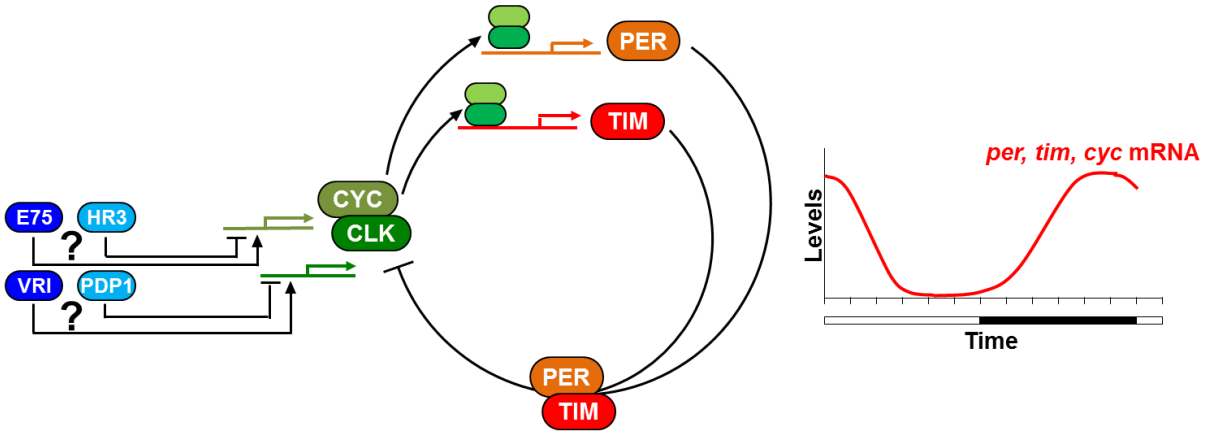

Fig. 6-1. The molecular mechanism of the *G. bimaculatus* clock.

CYC-CLK probably binds to E-boxes of *Gb'tim*, *Gb'E75*, and *Gb'per* to activate their transcription, and *Gb'*TIM and *Gb'*E75 increase during late day. TIM-PER heterodimer enters the nucleus, and suppresses transcription of *Gb'per* and *Gb'tim* by inactivation of CYC-CLK transcriptional ability during early night.

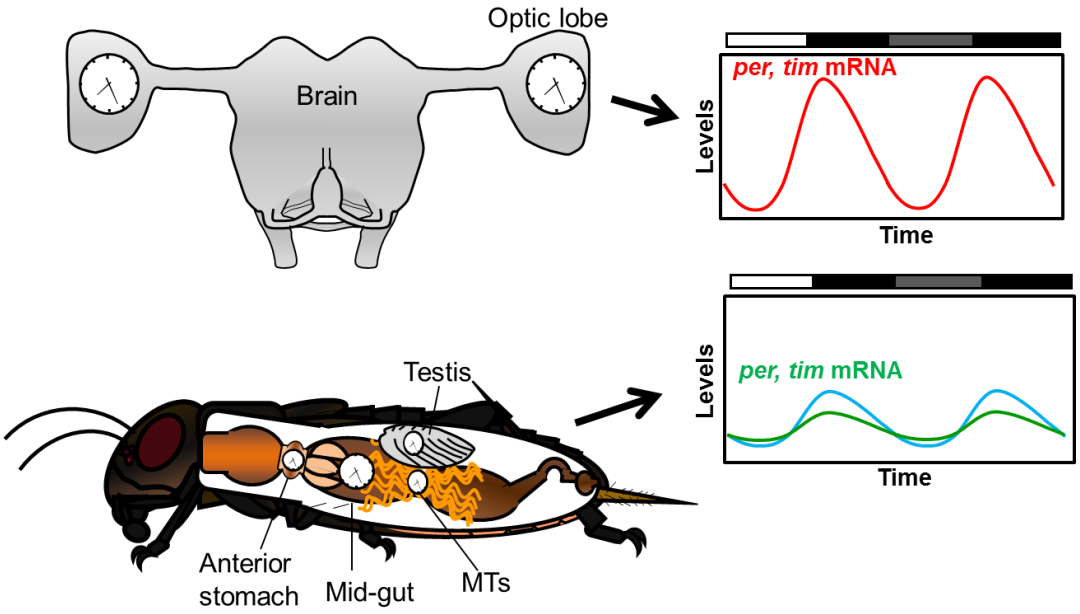

Fig. 6-2. Central and peripheral clocks in crickets

The cricket's central clock is located in the optic lobe in the brain. The central clock controls overall rhythms such as activity, feeding and mating. In contrast, there are clocks in many body parts, so-called peripheral clocks, which assign circadian rhythmicity with their specific functions. The molecular oscillations of clock genes are normally robust in the central clock, whereas ones in the peripheral tissues vary, suggesting different oscillatory mechanisms among tissues.

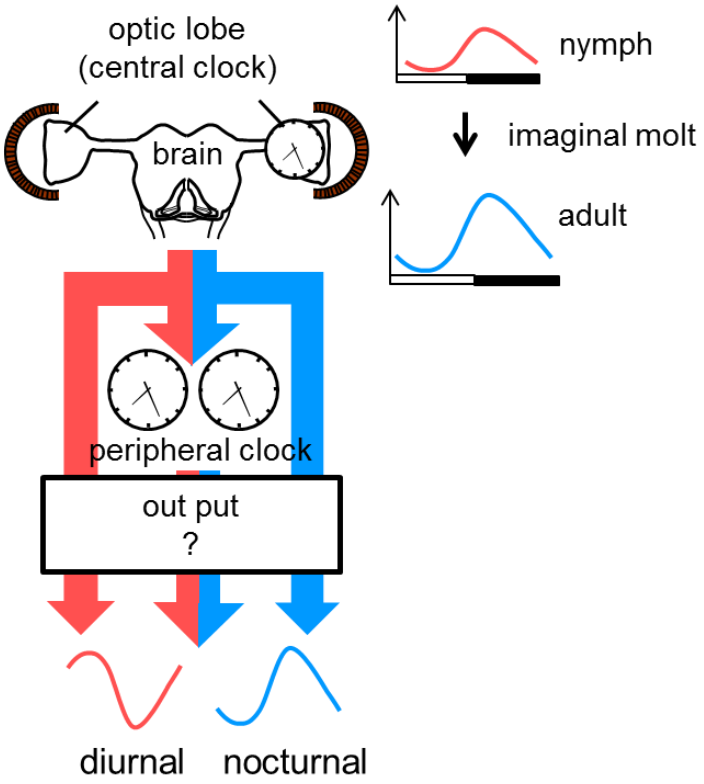

Fig. 6-3. The changes outside the optic lobe are related to the phase reversal. The nymphal and adult crickets *G.bimaculatus* have a central clock in the optic lobe that regulates overt activity rhythms and secondary oscillators in the tissues outside the optic lobe. In the nymphal optic lobe mRNAs of the clock genes were expressed with a pattern similar to those in adults but with significantly lower levels. The extra-optic lobe tissues showed a rhythmic expression in both nymphs and adults. The mRNA levels of clock genes in nymphal tissues were significantly lower than those in adults. The rhythms outside the optic lobe are weak in nymphs and may become robust after the imaginal molt. The rhythm reversal may depend on changes in the output pathways but not on that in the optic lobe clock.