# How clock genes affect the horn length in the Japanese rhinoceros beetle, *Trypoxylus dichotomus*?

Yuki Kato[1]*

[1]Todaiji-gakuen high school

## Abstract

Recent research revealed that circadian rhythm has a variety of functions. In this paper, I cloned cDNA of clock genes, *timeless* (*tim*) and *cryptochrome2* (*cry2*), and investigated the role of clock genes in the formation of the horn using RNA interference (RNAi) in *Trypoxylus dichotomus*. I tried to understand the circadian rhythm in *T. dichotomus*. Although I examined the temporal expression pattern of clock genes, did not obtain conclusive evidence that clock gene (*tim*, *cry2*) controls the circadian rhythm because Real-time PCR analysis did not show obvious rhythm. Real-time PCR analysis revealed that *tim* and *cry2* did not affect the length of the horn and prothorax. However, I noticed various insights about these clock genes.

**Key word:** clock gene, circadian rhythm, RNAi, *Trypoxylus dichotomus*, beetle horns

## 1. Introduction

Many organisms including insects have clock system and circadian rhythm. Molecular studies revealed that autoregulatory negative feedback loops consisting of so called "clock gene" constitute the clock system. Many insects have clock gene such as *period* (*per*), *timeless* (*tim*), *cryptochrome2* (*cry2*), *Clock* (*Clk*), and *cycle* (*cyc*). I show negative feedback control made by these clock gene in Fig. 1-1. However, all insects don't have the same clock system. *Drosophila* circadian rhythm does not use cry2. It is reported that RNAi of *tim* gene does not disrupt circadian locomotor rhythms in the cricket *Gryllus bimaculatus* (Danbara *et al*., 2010). *Apis mellifera* genome does not encode *tim* (Rubin *et al.,* 2006). On the other hand, some insects use both *cry2* and *tim* (Table1). It is reported that circadian rhythm has a variety of functions. The recent discovery shows that clock gene even affects body formation (Numata *et al.,* 2015).

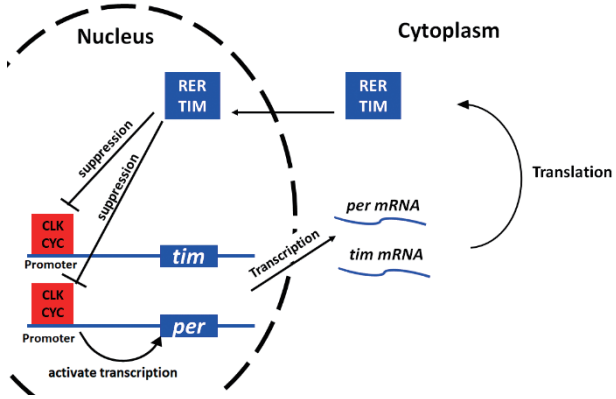


**Fig. 1-1.** An example of negative feedback loops (Drosophila melanogaster).

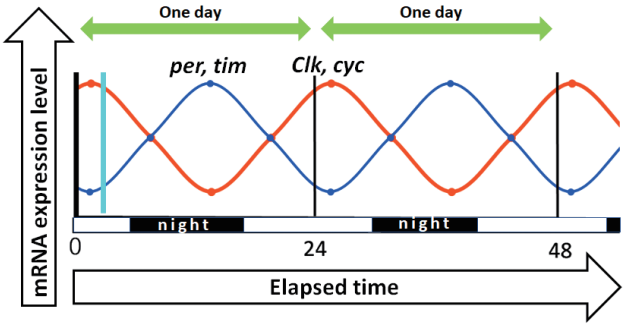


**Fig. 1-2.** Image of a change in the time of the clock gene mRNA abundance.

In this study, I aimed to understand the role of clock gene in trait formations such as length of horns. In order to know whether clock genes are involved in the circadian rhythm, I first examined the temporal expression pattern of clock genes (*tim* and *cry2*) in *T. dichotomus*. Second, I tried to find out the role of clock genes (*tim* and *cry2*) in trait formation such as length of horns in *T. dichotomus* by RNAi-mediated gene depletion.

Corresponding Researcher:
Takahiro Ohde (Graduate School of Agricultural Science, Kyoto University)
ohde.takahiro.4n@kyoto-u.ac.jp

**Table 1** Clock genes in insects (modified from Numata, 2014).

| Species name | Positive regulator actor | Negative regulator factor |
|---|---|---|
| *Gryllus bimaculatus* | CLOCK, CYCLE | PERIOD, TIMELESS |
| *Tribolium castaneum* | CLOCK, CYCLE | PERIOD, TIMELESS, CRYPTOCHROME2 |
| *Drosophila pseudoobscura* | CLOCK, CYCLE | PERIOD, TIMELESS |
| *Danoaus plexippus* | CLOCK, CYCLE | PERIOD, TIMELESS CRYPTOCHROME2, |
| *Aptheraea pernyi* | CLOCK, CYCLE | PERIOD, CRYPTOCHROME2 |
| *Apis mellifera* | CLOCK, CYCLE | PERIOD, CRYPTOCHROME2 |

## 2. Understanding the circadian rhythm in *Trypoxylus*: temporal expression pattern of clock genes (*tim* and *cry2*)

### 2-1 Result

I examined temporal expression pattern of clock genes to know if clock genes show circadian oscillation in *T. dichotomus*. I extracted total RNA from the larval leg of three individuals three times a day (14:30, 19:30, 7:00) and checked a change of the clock gene mRNA expression level.

I found that *tim* expression level decreases monotonously from 14:30 to 7:00 in all individuals. On the other hand, the characteristics of the way of change of *cry2* expression level differed among individuals. Regarding individual 2, *cry2* expression level decreases from 14:30 to 19:30 and changed little from 19:30 to 7:00. Regarding individual 3, cry2 expression level increases from 14:30 to 19:30 and declines from 19:30 to 7:00 (Fig. 2-1)

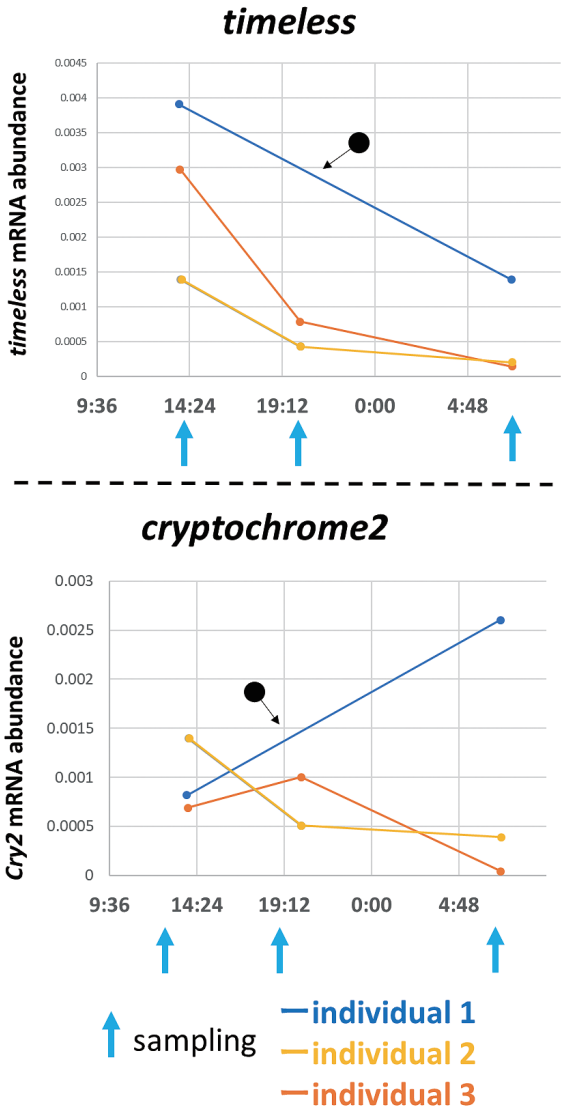


**Fig. 2-1.** The change of the clock gene mRNA abundance in a time course.

### 2-2 Discussion

Real-time PCR analysis did not detect obvious rhythm.

We have two hypotheses.

Hypotheses 1 is that *T. dichotomus* clock system does not need *cry2*. *tim* expression level changed similarly while *cry2* expression level changed differently in each individual (Fig. 2-1). It might be because larva gets information of time in some way, although larva lives in the underground, and the *tim* expression shows a circadian oscillation (Fig. 2-2 ❶).

Hypothesis 2 is that *cry2* expression level changes in different cycles among individuals (Fig. 2-2 ❷). Individual 2 and 3 changed as Fig. 2-1. So, I expected that each individual circadian clock has different cycles because larvae lives underground.

To check whether these hypotheses are correct, I need more data.

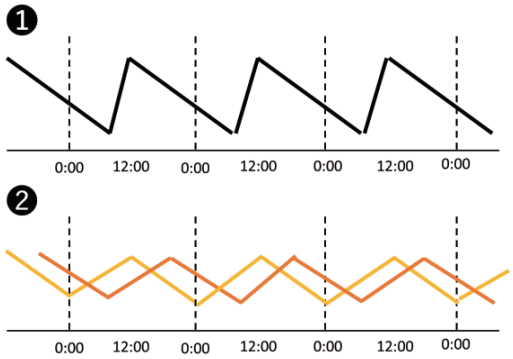


**Fig. 2-2.** Models for temporal expression pattern of the clock gene mRNA abundance.

## 3. The role of clock gene in trait formations such as length of horns

### 3-1 Result

I knocked down expression level of *tim* and *cry2* using

RNAi to know whether *tim* and *cry2* gene affects traits such as length of horns.

In beetles injected with double-stranded RNAs, targeted mRNA expression level was reduced (Fig. 3-1.).

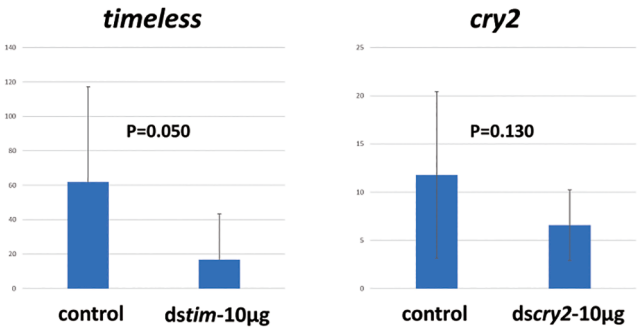


**Fig. 3-1.** Effect of RNAi.

I measured the length of horn and prothorax in RNAi-treated beetles. I measured the length of prothorax as an index of the size of whole body.

There was no significant difference in the size of either the horns or prothorax between control and RNAi-treated beetles (Fig. 3-2).

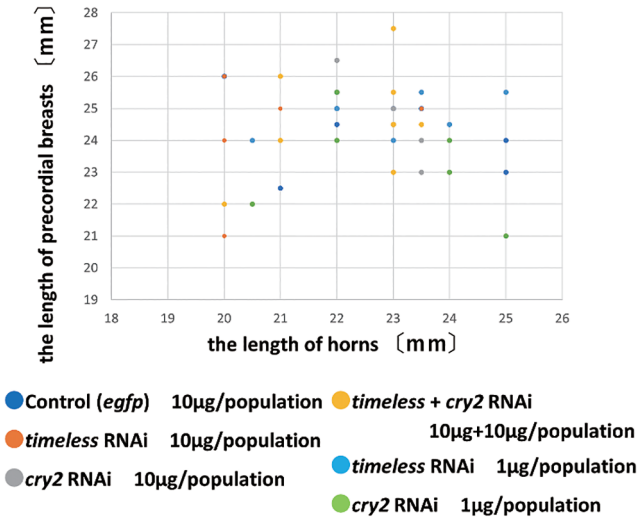


**Fig. 3-2.** Horn and prothorax lengths in RNAi-treated beetles.

I examined correlation between the value of "the length of horns/the length of prothorax" and the expression level of either *tim* or *cry2* to check whether the horn particularly grows or not. I knocked down expression level of either *tim* or *cry2* using RNAi. I checked the value of "the length of horns/the length of prothorax" and the expression level of either *tim* or *cry2* for both control and RNAi-treated beetles. Correlation coefficient concerned with *tim* was 0.1469, and correlation coefficient concerned with *cry2* was 0.0676. So, I can say safely that there was almost no correlation between the value of "the length of horns/the length of prothorax" and the proportion of either *tim* or *cry2*. (Fig. 3-3)

From our data, *tim* and *cry2* probably did not affect length of horns and prothorax.

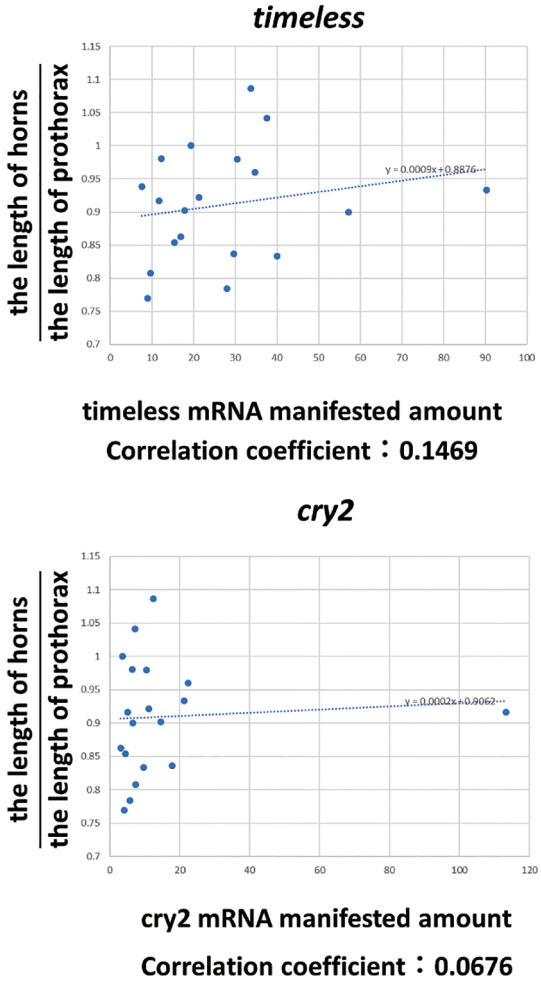


**Fig. 3-3.** Correlation between the horn length and mRNA expression level of clock genes.

### 3-2 Discussion

When I knocked down expression level of *tim* and *cry2* using RNAi, the length of horns and prothorax did not change. So, *tim* and *cry2* did not affect length of horns and prothorax.

From this result, it is likely that the beetle development does not depend on a biological clock, because larva lives underground. It may be possible that *tim* and *cry2* are involved in other body structure formations including the internal structure of the horn. I need further study to address this point.

## 4. Side story ~leg regeneration~

I had cut larval legs in order to get cDNA. When the larva became adult, I found that their leg is completely formed (Fig. 4.).

This result suggests that the beetle has ability to regenerate a leg, or that the beetle remakes a leg during the pupal period.

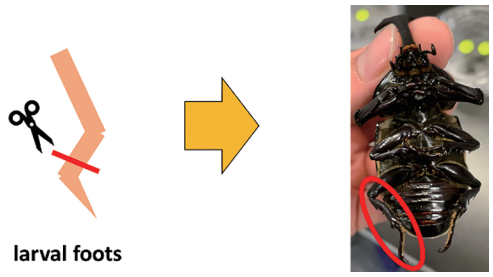


**Fig. 4.** Complete formation of an adult leg after cutting a larval leg.

## 5. Methods

### Materials

All *Trypoxylus* larvae used in this study were purchased from Kuwagata Kobo Mushikichi (Fukuoka, Japan).

### i) Synthesis of Double Stranded RNA

(1) I synthesized cDNA from brain, leg muscle tissue, fat, hemolymph. (2) I designed gene-specific primers with T7 promoter sequence at 5′ end by using Primer3 software. (3) I amplified the target DNA sequence by PCR. (4) RNAs were *in vitro* transcribed from PCR products as templates. Then + and - strand RNAs were purified and annealed.

### What is RNAi (RNA interference)

RNAi has a function of suppressing the amount of mRNA. In this article, I show the mechanism of RNAi. First, I injected Double Stranded RNA. Then, Dicer, a type of RNA degrading enzyme, cut and transformed it into siRNA which is 22-25bp. Following that, one strand of siRNA was incorporated into RISC (RNA-induced silencing complex). Finally, RISC cut mRNA which has some sequence as siRNA. That is why RNAi has a function of suppressing the amount of mRNA. (Fig. 5) (modified from Tajima, 2013)

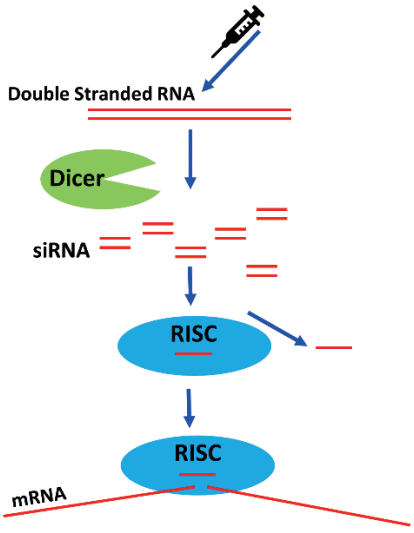


**Fig. 5.** Mechanism of RNAi.

### ii) Injection of Double Stranded RNA

I injected double stranded RNA into beetle larvae. Spots we injected were boundaries between head and thorax. Our design of larval RNAi experiment is shown in Table1. I separated into different groups.

**Table 2** Design of larval RNAi experiment.

| Double stranded RNA | Amount of injected RNA / individual | No. of injected individual |
|---|---|---|
| Control (*egfp*) | 10 μg | 10 |
| *tim* | 10 μg | 10 |
| *cryptochrome2* | 10 μg | 10 |
| *tim* + *cry2* | 10 μg+10 μg | 10 |
| *tim* | 1 μg | 8 |
| *cry2* | 1 μg | 7 |

### iii) Sampling

One week after injecting the double stranded RNA, I cut larva's legs. The sample was stored in TRIzol.

I extracted total RNA and generated a cDNA by a reverse transcriptase.

### iv) Measurement of horns

After the larva becomes an adult, I measured the length of horn and prothorax with a threat.

### v) qPCR

I measured mRNA abundance by qPCR. I amplified the target sequence from the leg cDNA. I used *RpL32* as a reference gene.

I got a Ct level (2nd Derivative Maximum). The Ct level is defined in the next expression.

I define the Amplification curve as C: $y = f(x)$

$\mathrm{Max} f''(x) \Leftrightarrow x = ct$ (2nd Derivative Maximum)

I performed melting curve analysis after the amplification in order to see whether the sequence that I did not expect was not amplified. (Fig. 6.) As a result, I amplified only the sequence that I expected.

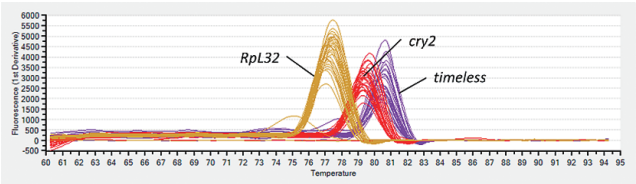


**Fig. 6.** Melting curve analysis.

### vi) Data processing

I handled a Ct level in the following expressions and measured the relative value of mRNA.

I can express the amount of the PCR product in the following expressions.

$$p \times 2^n \quad (p\text{: } initial\ amount)\ (n\text{: } cycle)$$

I define the initial amount of the target gene as “a”, “ct” of the target gene as “$ct_a$”, the initial amount of reference gene as “b” and “ct” of reference gene as “$ct_b$”

$$a \times 2^{ct_a} = b \times 2^{ct_b}$$

$$\therefore ①② \ \Delta ct = ct_a - ct_b \Leftrightarrow b = a \times 2^{\Delta ct}$$

$$\Delta\Delta ct = \Delta ct - \Delta ct_m$$

$2^{-\Delta\Delta ct}$ → the relative value of mRNA

## Conclusions

The purpose of this study is to find out the role of circadian rhythm in the expression level of clock gene and to investigate the role of clock gene in trait formations such as length of horns.

I cannot obtain conclusive evidence that clock gene (*tim*, *cry2*) controls the circadian rhythm in *T. dichotomus.*

And I can know *tim* and *cry2* did not affect traits such as length of horns and precordial breasts in *T. dichotomus*.

However, I was able to notice various insights about clock gene.

## Acknowledgement

I appreciate to T. Ohde for assistance and support. I thank H. Numata for teaching insect clocks. I thank all staff members who supported me. This study was supported by ELCAS.

## References

Numata H. Insect clocks. Hokuryukan. (2014).

Tajima S. Epigenetics. Kagaku-Dojin. (2013).

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

Rubin E. B., Shemesh Y., Cohen M., Elgavish S., Robertson H. M. and Bloch G. 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 Res.* **16**, 1352–1365 (2006).

Numata H., Miyazaki Y. and Ikeno T. Common features in diverse insect clocks. *Zool. Lett.* **1**, 10 (2015).

# 時計遺伝子がカブトムシの角の長さなどの形質形成にどれほど影響を与えるか

加藤 祐基[1]*

[1] 東大寺学園高等学校

*ELCAS11th 個人型 応用昆虫生理学：最強のカブトムシ飼育プロトコルを作ろう！

## 要旨

最近の研究で，概日リズムにはさまざまな機能があることが分かってきた．本研究では，時計遺伝子〔*timeless (tim)* および*cryptochrome2* (*cry2*)〕のcDNAをクローニングし，RNA干渉を利用して，カブトムシの角の長さなどの形質形成における時計遺伝子の役割を調べた．

カブトムシの時計遺伝子（*tim* および *cry2*）の発現パターンを調べることで，体内時計の存在の有無を検証した．

定量PCR分析で，明らかな発現量のリズムが示されなかったため，時計遺伝子（*tim*，*cry2*）がカブトムシの概日リズムを制御しているという決定的な証拠を得ることができなかった．また，定量PCR分析により，*tim* および *cry2* は，カブトムシの角の長さや前胸などの形質形成に影響を与えないことがわかった．

カブトムシの角の形成が，概日リズムに頼らないのは，幼虫が土の中で生活しているからだと考えた．しかし，角の内部構造を含むその他の体の構造に*tim* と *cry2* が関与している可能性があると考えられる．これらを確かめるには，更なる研究が必要だ．

**重要語句：**時計遺伝子，概日リズム，RNAi，カブトムシ