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Supplementary Information for

Photoperiod and temperature separately regulate nymphal development through JH and insulin/TOR signaling pathways in an insect

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Supplementary Information Text

SI Materials and Methods

cDNA cloning

Total RNA was extracted with TRIzol® Reagent (Ambion, Austin, TX, USA) from 10 heads of 2nd instar nymphs collected at zeitgeber time (ZT) 6. A 4.5 µg sample of total RNA was used for reverse transcription to obtain cDNA, using the PrimeScript® RT Reagent Kit (Takara, Otsu, Japan). Using the single-stranded cDNA as a template, we performed PCR with the primers shown in Table S1. The PCR conditions employed were 30 s for denaturation at 95 °C, 30 s for annealing at 55 °C, and 1 min 30 s for extension at 72 °C for 40 cycles with EmeraldAmp® PCR Master Mix (Takara). Sequences were analyzed by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Measurement of mRNA levels

Total RNA was extracted and purified from 3 heads of *Modicogryllus siamensis* with TRIzol Reagent (Ambion). The head samples included the CC-CA complex. Approximately 4.5 µg of total RNA of each sample was reverse transcribed with random 6mers using a PrimeScript RT Reagent Kit (Takara). qPCR was performed using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and a KAPA SYBR FAST qPCR Kit (NIPPON Genetics, Tokyo, Japan), including SYBR Green with the gene specific primers (Table S1). qPCR conditions were as follows: 95 °C for 20 s and then 40 cycles of 95 °C for 3 s, 60 °C for 30 s with a 0.4 µM concentration of each primer. The results were analyzed using the software associated with the instrument. The values were normalized with the values of *Ms'rp49* (LC458936), a housekeeping gene. Results of 3 or 4 independent experiments were pooled to calculate the mean ± SEM.

RNA interference.

Double-stranded RNA (dsRNA) of *Ms'myo*, *Ms'Inr,* and *DsRed2* was synthesized using MEGAscript® High Yield Transcription Kit (Ambion). *DsRed2* is a variant of the red fluorescent protein gene (*DsRed*), derived from a coral species (*Discosoma* sp.), and is lacking in the cricket's genome. Template cDNA fragments for *in vitro* transcription were amplified by PCR from the cricket brain cDNA library using ExTaq DNA polymerase (Takara). Primers tagged with T7 or T3 promoter sequences were used for PCR amplification (Table S1). For *DsRed2* dsRNA, a *DsRed2* cDNA fragment was amplified from pDsRed2-N1 (Clontech, Mountain View, CA, USA) with primers listed in Table S1. Amplified fragments were extracted with phenol/chloroform, precipitated with ethanol, and resuspended in Ultra-Pure Water (Invitrogen). Amplified fragments were purified with phenol/chloroform and precipitated with ethanol. RNA was synthesized from each of these cDNA fragments using T7 or T3 RNA polymerase. Synthesized RNA was extracted with phenol/chloroform, and suspended in 50 µl TE buffer after isopropanol precipitation. The yield and quality of RNA were assessed by absorbance with a spectral photometer (Genequant Pro, Amersham Biosciences, Piscataway, NJ, USA) and the same amount of sense and antisense RNA was mixed. The RNAs were denatured for 5 min at 98 °C and annealed by a gradual cool-down to room temperature. After ethanol precipitation, the obtained dsRNA was suspended in Ultra-Pure Water and adjusted to a final concentration of 10 µM. The dsRNA solution was stored at -80 °C until use.

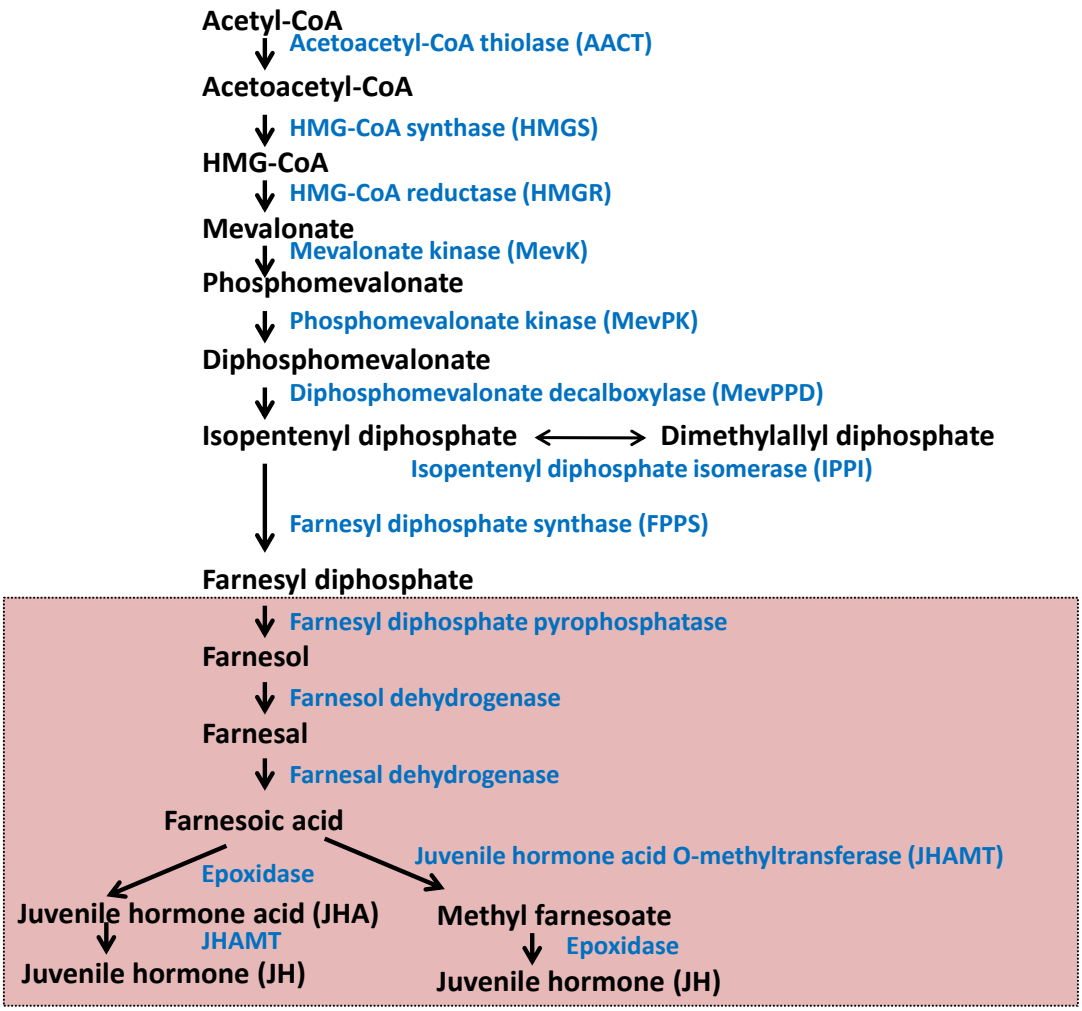

Fig. S1. Juvenile hormone (JH) biosynthesis pathway. The biosynthesis pathway can be divided into two parts, i.e., the early step, the mevalonate pathway (MVAP) and the late step, the juvenile hormone branch. The MVAP pathway produces farnesyl diphosphate from the acetyl-coA through several enzyme-mediated processes. In the JH branch, JH is produced through several enzymatic reactions from farnesyl diphosphate. The final two steps differ among insect species but JHA or farnesoic acid is converted to JH through a juvenile hormone acid O-methyltransferase (JHAMT)-mediated process. Metabolites are shown in black letters and enzymes in blue. Adopted from Noriega (1).

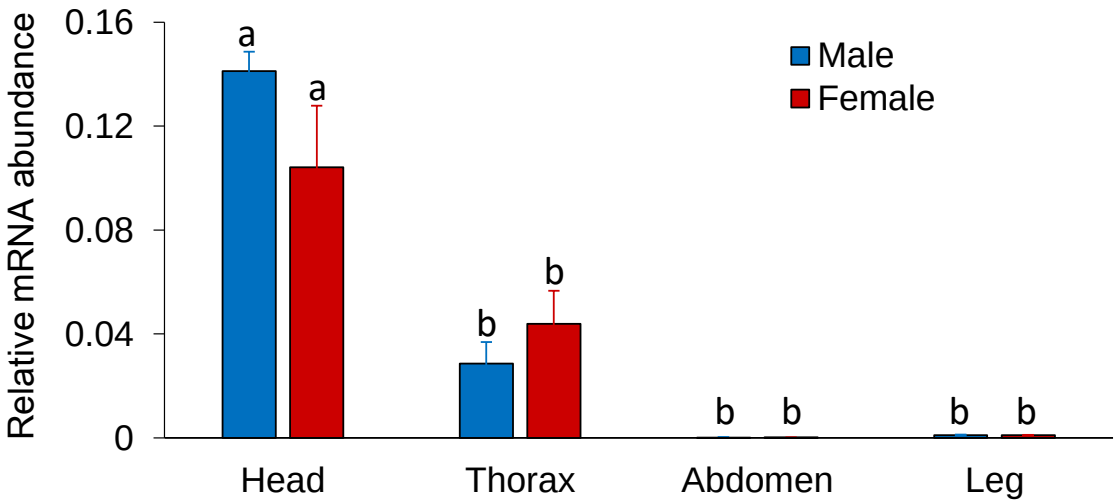

Fig. S2. Relative abundance of *Ms'myo* mRNA in the head, thorax, abdomen, and leg of last-instar nymphs at 6 h after light-on (ZT6). Blue and red columns indicate male and female crickets, respectively. Values with different lowercase letters significantly differ from each other (Tukey test, $P < 0.05$). The abundance of *Ms'myo* mRNA was measured by quantitative real-time RT-PCR with total RNA extracted from the respective tissues. The abundance of *Ms'rp49* mRNA was used as an internal reference. The data collected from four independent samples were averaged and plotted as means ± SEM.

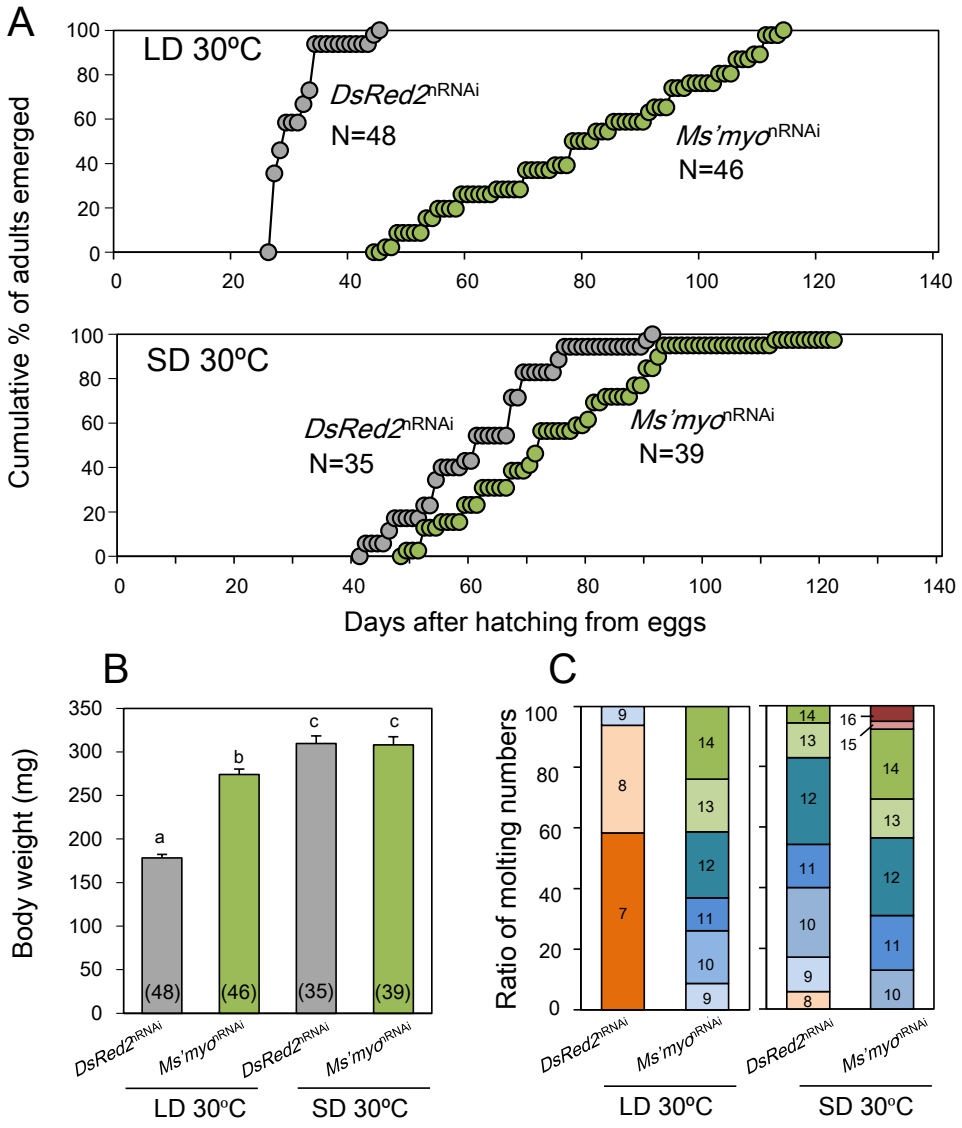

Fig. S3. The effects of *Ms'myo* nymphal RNAi (*Ms'myo*^{nRNAi}) on nymphal development at 30 °C. **A:** Adult emergence patterns under long day (LD) and short day (SD). Gray and green symbols indicate *DsRed2*^{nRNAi} control and *Ms'myo*^{nRNAi}-treated crickets, respectively. N indicates the number of animals used. **B**: Adult body weight of *DsRed2*^{nRNAi} control (gray) and *Ms'myo*^{nRNAi}-treated crickets (green) under LD and SD. Error bars indicate SEM. Values with different lowercase letters significantly differ from each other. **C**: Molting numbers of *DsRed2*^{nRNAi} control (gray) and *Ms'myo*^{nRNAi}-treated crickets (green) under LD and SD. Numbers indicate those molting number. *Ms'myo*^{nRNAi} significantly delayed adult emergence (Mann-Whitney's U-test, $P < 0.001$) and increased molting numbers compared with that of *DsRed2*^{nRNAi} crickets (Mann-Whitney's U-test, $P < 0.001$) under both LD and SD conditions. Body weights were significantly increased in *Ms'myo*^{nRNAi}-treated crickets under LD (ANOVA followed by Tukey test, $P < 0.05$). Values with different lowercase letters significantly differ from each other.Type or paste legend here. Paste figure above the legend.

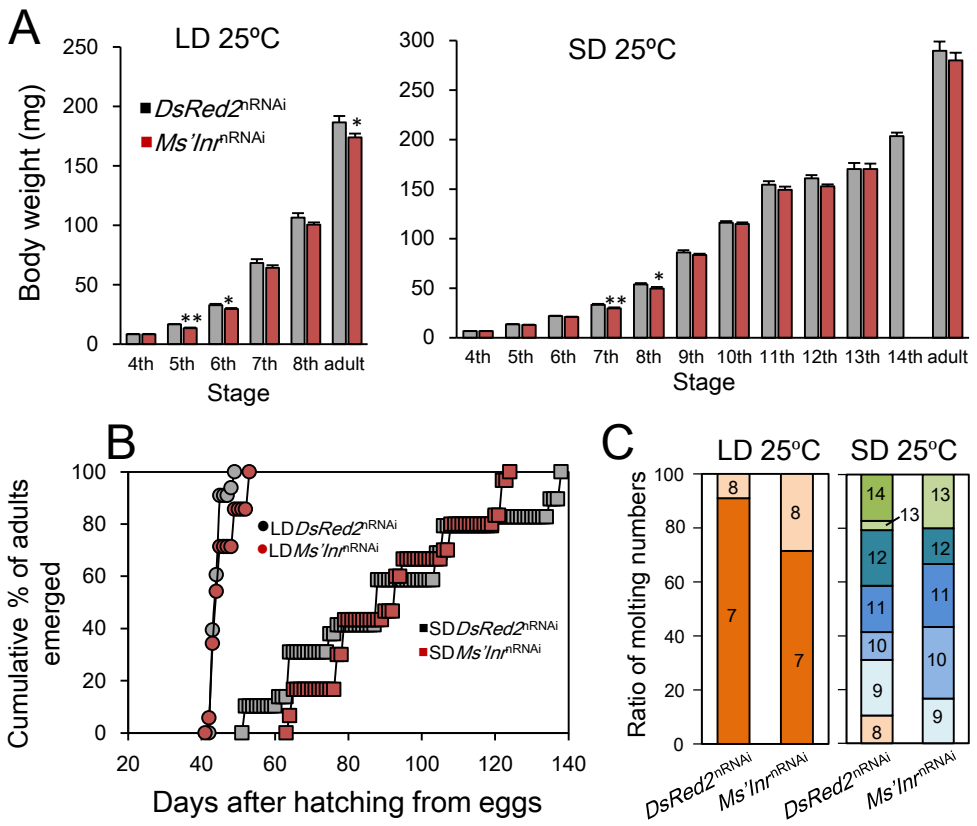

Fig. S4. Effects of *Ms'Inr*^{nRNAi} on the nymphal development under long day (LD) and short day (SD) conditions at 25 °C in the cricket *Modicogryllus siamensis*. The number of *Ms'Inr*^{nRNAi} and *DsRed2*^{nRNAi} animals used were 35 and 33 for LD and 30 and 29 for SD, respectively. **A**: Suppression of the growth rate by *Ms'Inr*^{nRNAi}. Under both LD and SD, *Ms'Inr*^{nRNAi}-treated crickets (brown column) showed less of an increase in body weight than did *DsRed2*^{nRNAi}-treated controls (gray column). Error bars indicate SEM. **,$P < 0.01$, *, $P < 0.05$, *t*-test. **B**: Adult emergence under LD and SD. Brown and gray symbols indicate *Ms'Inr*^{nRNAi}-treated and *DsRed2*^{nRNAi}-treated crickets, respectively. Circles and squares indicate results under LD and SD, respectively. Slight but not statistically significant delays in adult emergence was observed in *Ms'Inr*^{nRNAi}-treated crickets under LD conditions. No apparent change was observed under SD conditions. **C**: Molting numbers of *Ms'Inr*^{nRNAi}-treated and *DsRed2*^{nRNAi}-treated crickets. The numbers in columns indicate number of moltings. Under LD conditions, *Ms'myo*^{nRNAi} significantly increased the ratio of crickets with 8 moltings (*U*-test, $P < 0.05$), but never induced a 9th or later molting. No significant changes were induced under SD conditions (*U*-test, $P > 0.79$).

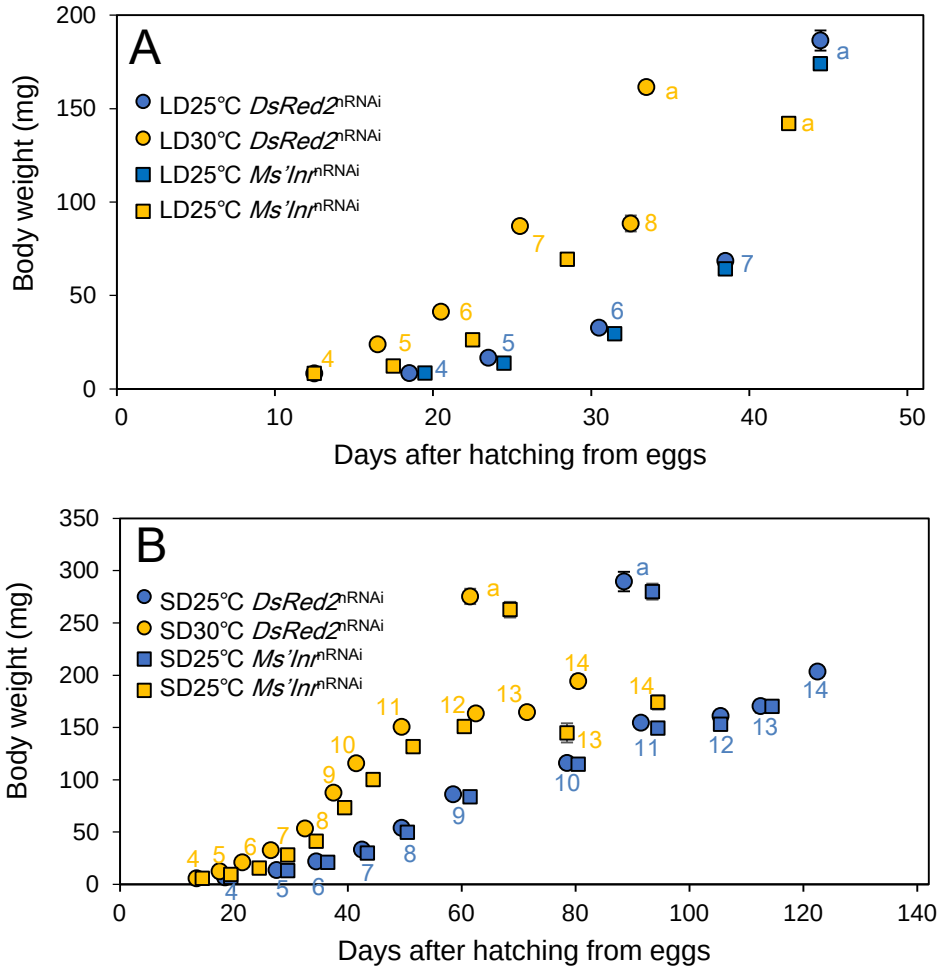

Fig.S5. Nymphal developmental rate of the cricket *Modicogryllus siamensis* treated with dsRNA of *DsRed2* (*DsRed2*^{nRNAi}, circle) or *Ms'Inr* (*Ms'Inr*^{nRNAi}, square) on day 1 of the 4th instar nymphal stage. Body weight at 25 °C and 30 °C for long-day (LD)(A) and short-day (SD)(B) conditions are shown with blue and orange symbols, respectively. Data shown in Fig. 4 and Fig. S4 are replotted. Average body weight is plotted with SEM against the day at which 50% of the respective instar nymphs molted. Numbers beside the symbols indicate instar number. Adults include those that underwent a different number of molts, thus the value (a) plotted at the day when half of them became adult. Note that growth rate as measured with body weight was much higher at 30 °C than at 25 °C under both LD and SD, and that *Ms'Inr*^{nRNAi} treatment suppressed the growth rate and delayed nymphal development, especially at 30 °C.

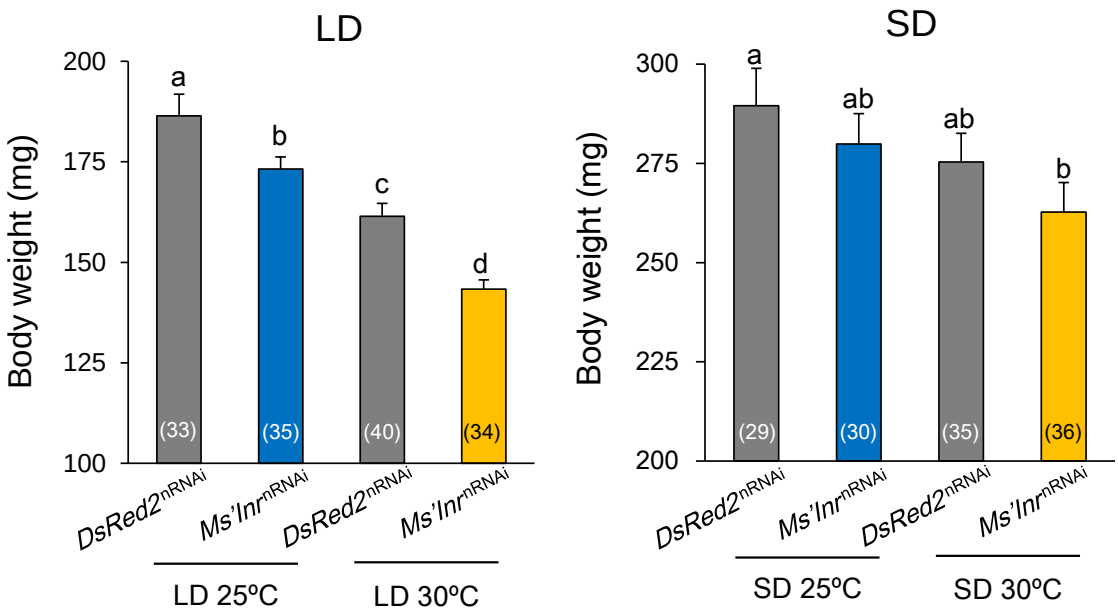

Fig. S6. Comparison of adult body weight among crickets treated with *DsRed2*^{nRNAi} or *Ms'Inr*^{nRNAi} kept at 25 °C or 30 °C under long-day (LD) or short-day (SD) conditions. Data are replotted from Fig. 4 and Fig. S4. Values with different lower case letters significantly differ with each other (ANOVA followed by Fisher's LSD, $P < 0.05$). Numbers in parenthesis indicate number of animals used. Error bars indicate SEM. The body weight was smaller in *Ms'Inr*^{nRNAi}-treated crickets than *DsRed2*^{nRNAi}-treated control crickets. It was also always greater at lower temperature (25 °C). However, the difference was significant under LD conditions, whereas under SD conditions, difference was significant only between *DsRed2*^{nRNAi} at 25 °C and *Ms'Inr*^{nRNAi} at 30 °C.

Table S1. Sequences of primers used for cDNA fragment cloning, quantitative real-time RT-PCR, and double stranded RNA synthesis.

gene	Forward (5'-3')	Reverse (5'-3')
For PCR		
Ms'myo	TCCGACAACAAGAAGCACC	ACTCTCCGCCAACTGTGATG
Ms'jhamt	CAGGACGTCTGCTGCCATAT	CGCAGGATAAATAGCCAGGC
Ms'Kr-h1	CTCCAAGACCTTCATCCAGAGC	TGCACTTGTACACCTTCTCTCC
Ms'llp	TCGCCACAAGTCGCAGATC	ACCCTGTCTCTTTTGGCGAT
Ms'lnr-f1	AAAACCGCAGCTGCTTTTGT	ATGGCATGGCGTACTTGTCT
Ms'lnr-f2	CCCCAAATAAGCTGCGTGATG	ACAAGCTCAGCCAATGCAGG
Ms'Tor	TGTGTTCCGTCGCATCTCAA	GTCGTACACAAAGGCCTCCA
For real-time PCR		
Ms'myo	AAGAATTTGGCTGGGACTGG	TGTGCGTGTGAGGGTATTTC
Ms'jhamt	CACAAAAATGCGCGGTGTTC	TCGGCAAAAGTAGTGTCCAC
Ms'Kr-h1	TGCTCCAAGACCTTCATCCAG	TGTGCACCTTGAGTTGCTTG'
Ms'llp	TGGGCGATACCTGTCTAACATG	AGCTGCTCTTCAACAGATGC
Ms'lnr	CCCCAAATAAGCTGCGTGATG	TTGTCATGGGAGTGAGAGAGTG
Ms'Tor	AGCAGAGACCTCGCAAATTG	TCCAAACAGCTGCATAACCC
Ms'rp49	CATTGGCTATGGAAGCAACAA	TCATCAACACTTCCAACTCCTTTAC
For dsRNA synthesis		
ds*Ms'myo*	TAATACGACTCACTATAGGGTACCATCGGTCTCAATTGC	AATTAACCCTCACTAAAGGGAACCGCATCTATCGACAACC
ds*Ms'lnr*	TAATACGACTCACTATAGGGTGGTAAAAGGCCCTTGGTCC	AATTAACCCTCACTAAAGGGGGTGTTGTCTCACAAGGGCA
ds*DsRed2*	TAATACGACTCACTATAGGGTCATCACCGAGTTCATGCG	TAATACGACTCACTATAGGGCTACAGGAACAGGTGGTGGC

References

1. F. G. Noriega, Juvenile hormone biosynthesis in insects: what is new, what do we know, and what questions remain? *Int. Schol. Res. Notices* 2014:16 (2014).