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Source: Zoological Science, 34(2) : 151-160

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zs160153

ZOOLOGICAL SCIENCE **34**: 151–160 (2017)

Clock and Hormonal Controls of an Eclosion Gate in the Flesh Fly *Sarcophaga crassipalpis*

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The eclosion gate in insect development is controlled by the circadian clock and hormonal cascade. To study mechanisms underlying the eclosion gate, we examined eclosion-timing signals from the circadian clock, and the role of 20-hydroxyecdysone in the eclosion gate of the flesh fly, *Sarcophaga crassipalpis*. Phase responses of the eclosion rhythm were examined by applying a low-temperature pulse in the day prior to the first eclosion peak. A low-temperature pulse applied about 5.4 h before eclosion advanced an eclosion peak by 0.9 h. This indicates that an interval from the zeitgeber (external environmental cues) input to the behavioral output by the circadian clock is 4.5 h. Signals released by the circadian clock in the last 4.5 h before eclosion could change eclosion time. In the prothoracic gland, daily changes in immunoreactivity against a circadian clock protein PERIOD were observed in the last two days before eclosion. Hemolymph titers of 20-hydroxyecdysone were very low in the last two days of the pupal period. 20-hydroxyecdysone injections caused a delay, not an advancement, in eclosion time in a time dependent manner: pharate adults were sensitive to 20-hydroxyecdysone about 20 and 16 h before eclosion, whereas no significant effects were observed about 12 and 8 h before eclosion. These results suggest that 20-hydroxyecdysone is not a timing signal submitted by the circadian clock but an indicator to suppress premature eclosion. The circadian clock in the prothoracic gland presumably sends a signal distinct from ecdysteroids from several hours before eclosion to time the onset of eclosion.

Key words: circadian clock, prothoracic gland, population rhythms, PERIOD immunocytochemistry, 20-hydroxyecdysone, *Sarcophaga crassipalpis*

INTRODUCTION

In many insects, ecdysis, such as egg-hatching, pupation, and eclosion, each of which takes place once in the life cycle, occurs at a certain time of the day. In populations of the fruit fly *Drosophila pseudoobscura* (Diptera: Drosophilidae), eclosion activities occur in bursts in the hours following dawn in a rhythmic manner under light-dark conditions, and the population rhythm continues with an endogenous period of approximately 24 h under constant dark conditions (Pittendrigh, 1954). Eclosion activities are allowed in a certain time of day, and Pittendrigh (1965) and Pittendrigh and Skopik (1970) have called this allowed time the "gate" and proposed that an endogenous circadian clock sets the eclosion gate to a certain time of day. Other than the adult eclosion rhythm, it is known that egg-hatching and pupation in some insects such as the mosquitoes, *Aedes vittatus* and *A. aegypti* (Diptera: Culicidae), are also under circadian control (Saunders, 2002). Although the circadian ecdysis rhythm has been reported in many species, the molecular mechanisms underlying the circadian-ecdysis gate have not been well understood. In the gating mechanism, circadian clocks and hormonal events for development have to act in concert to determine ecdysis timing (Di Cara and King-Jones, 2013).

In *D. melanogaster*, circadian clock neurons residing in the optic lobe of the brain called small ventral lateral neurons (s–LN_vs) and peripheral clock cells in the prothoracic gland (PG) are both involved in driving eclosion rhythms (Myers et al., 2003). s–LN_vs containing a neuropeptide pigment-dispersing factor (PDF) are necessary not only for maintaining the eclosion rhythm but also for oscillating the clock gene expression in the PG. In the saturniid moth *Samia cynthia ricini* (Lepidoptera: Saturniidae), the population rhythm of the gut purge characteristic of larval-prepupal transition may be controlled by the PG circadian clock (Mizoguchi and Ishizaki, 1982).

The hormonal cascade triggering larval or pupal ecdysis behavior has been well studied in the tobacco hornworm *Manduca sexta* (Lepidoptera: Sphingidae) and *D. melanogaster* (Žitňan et al., 2007). Pre-ecdysis triggering hormone and ecdysis triggering hormone (ETH) secreted from the epitracheal gland and eclosion hormone (EH) are triggering factors of a subsequent hormonal cascade for ecdysis behavior. Ecdysteroids from the PG are also key hormones for it. In *M. sexta*, increasing titers of ecdysteroids between 48 and 30 h before pupal ecdysis stimulate the epitracheal gland cells to express *eth* (Žitňan et al., 1999). Further, the ecdysteroid receptor is present in the upstream region of *eth*. A successive decrease

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doi:10.2108/zs160153

in ecdysteroids is also necessary prior to ecdysis behavior for releasing ETH to the hemolymph in *M. sexta* and *D. melanogaster* (Truman et al., 1983; Robinow et al., 1993).

Studies on the hormonal cascade for eclosion behavior and the circadian clocks driving the eclosion rhythm have been performed independently, and it remains unknown how the circadian clock in the brain and PG affects the hormonal cascade triggering ecdysis, or *vice versa*, to determine the circadian gate. To study the circadian gating mechanism, it is necessary to examine both developmental and chronobiological aspects using the same species.

The flesh flies *Sarcophaga* spp. (Diptera: Sarcophagidae) have been used for chronobiological studies of circadian eclosion rhythm; circadian clock properties affecting eclosion rhythm and its responsiveness to light and temperature have been studied in *Sarcophaga argyrostoma* (Saunders, 1976, 1979); the possible involvement of circadian clock genes *period* and *timeless* in eclosion rhythms has been proposed in *Sarcophaga bullata* (Goto et al., 2006). In *Sarcophaga crassipalpis*, which has a clear circadian eclosion rhythm, thermoperiodic regulation of eclosion timing has been shown under natural and laboratory conditions (Yocum et al., 1994; Joplin and Moore, 1999; Miyazaki et al., 2011). However, the neural and endocrinological mechanisms for circadian eclosion rhythms remain unresolved.

With chronobiological knowledge, *Sarcophaga* spp. are suitable for analysis on mechanisms constituting a circadian eclosion gate. In the present study, we examined phase responses of the circadian eclosion rhythm of *S. crassipalpis* to find the last moment at which the circadian clock is able to reset eclosion time, in an effort to better understand the timing for when the circadian clock submits output signals for the onset of eclosion. We also examined a circadian clock protein PERIOD (PER) immunoreactivity in the PG, and effects of ecdysteroid injections on the determination of the eclosion timing to discuss roles of the PG and 20-hydroxyecdysone (20E) in the eclosion gate.

MATERIALS AND METHODS

Insects

Adults of *S. crassipalpis* Macquart were captured in Sapporo City (E 141°21′, N 43°04′) in 2010. Their progeny were kept under a temperature cycle of 12-h thermophase at 28 ± 1°C and 12-h cryophase at 22 ± 1°C (TC 12:12) in continuous light (LL, 0.15 W/m^2), or constant conditions at 25 ± 1°C or at 28 ± 1°C under LL. Adult flies were provisioned with water, sugar, and a piece of beef liver. Females laid larvae on a piece of beef liver (day 0), and the developing larvae then fed on it. All experiments were performed under LL.

Recording of eclosion rhythms

The recording method of eclosion activities was adopted from Watari (2002) for the study of *Delia antiqua* (Diptera: Anthomyiidae). The recording apparatus was made of a thick plastic plate flanked with an infrared-light emitter and a detector (EE-SPW321, OMRON, Kyoto, Japan) and was based on the "falling ball" principle (Truman, 1972). In this apparatus, each pupa was loaded into a hole (8.0 mm in diameter) and two types of balls (3.5 and 2.5 mm in diameter) were placed in front of each pupa. When a fly emerged, it pushed the large ball with its head and only the small ball fell in a groove crossing an infrared beam. When the ball crossed the infrared beam, a signal was fed to a computer and the number of eclosion events was counted every 6 min.

Analysis of rhythmicity

Rhythmicity in eclosion was analyzed by the parameter *R* (Winfree, 1970). Eclosion events were pooled for 3–5 days and the number of events per h was summed. An 8-h period (a hypothetical gate) of the day containing most of the events was determined. The measure *R* was calculated by dividing the number of events outside the 8-h period by the number of events within the 8-h and then multiplying it by 100. The theoretical range of *R* is from 0, when all events occur within the gate, to 200, if events were distributed uniformly through the day. *R* values of 90 or larger were considered arrhythmic. *R* values of 60 or less were considered as rhythmic, and those between 60 and 90 were weakly rhythmic (Smith, 1985).

Construction of phase response curves (PRCs)

Pupae were transferred from TC 12:12 to a constant temperature of 28 ± 1°C at the end of cryophase (zeitgeber time, ZT, 24) of day 16 and eclosion events were recorded by the recording system described previously. Medians of eclosion time in each peak were calculated and intervals between two serial peaks were averaged to compute a free-running period (τ). A 4-h low temperature pulse of 22 ± 1°C or a 1-h low temperature pulse of 4 ± 1°C was provided every 4 h in the last one or two days of pupae under the constant conditions. Amounts of phase shifts were calculated by time differences between the median of the eclosion peak without a temperature pulse and that with a temperature pulse (Supplementary Figure S1). The phase shift amounts were calculated as circadian time (CT), where the unit of time corresponds to 1/24 of each τ. As eclosion events occurred around the start of subjective days in *S. crassipalpis*, the time of the eclosion peak median under constant conditions without low-temperature pulses was set at CT 0. To construct PRCs, the amounts of phase shifts in CT were plotted as a function of CT phase at which the pulse was started.

Immunocytochemistry

PERIOD (PER) immunocytochemical staining was performed in the retrocerebral complex (RC) of male pupae collected every 6 h during the last two days of the pharate adults on ZT 0, 6, 12, and 18, and on ZT 0 and 6 on the day of adult eclosion by the ABC method (Vectastain ABC standard kit, Vector Laboratories, California, USA) under TC 12:12. Sex was determined by the copulatory organ, and age (one or two days before eclosion) of the pupae was determined by sharpness of thoracic stripes on the cuticle. The head with the anterior half of the thorax was fixed in 4% paraformaldehyde for 4 h at 4°C. After washing with 0.1-M phosphate-buffered saline with 0.1% Triton X-100 (PBST), the RC was dissected out and incubated in 3% H_2O_2 for 1 h at room temperature to reduce endogenous peroxidase activity. After blocking in PBST containing 0.5% bovine serum albumin (BSA) for 1 h, the RCs were kept in the primary goat anti-*D. melanogaster*-PER antiserum (sc-15720, Santa Cruz Biotechnology, California, USA) diluted at 1:1000 with 0.5% BSA in PBST for 2 days at 4°C. To test specificity of the antibody, the primary antibody solution preincubated overnight with 1 μg/ml blocking PER-peptides (sc-15720 P, Santa Cruz Biotechnology) was used for the staining. After washing with PBST, the RCs were kept in the secondary antiserum, donkey anti-goat immunoglobulin conjugated with biotin (705-065-003, Jackson ImmunoResearch Laboratories, Pennsylvania, USA), diluted at 1:200 with 0.5% BSA in PBST for 1 day at 4°C. The RCs were incubated in an avidin-biotin complex solution and diluted at 1:100 with PBST for 1 day at 4°C. After washing with PBST, these were preincubated with 3, 3′-diaminobenzidine (DAB, Sigma-Aldrich, Missouri, USA) in 0.1 M Tris-HCl for 1 h at 4°C and incubated with 0.03% DAB in 0.1 M Tris-HCl containing 0.01% H_2O_2 for approximately 10 min at room temperature. After washing with Tris-HCl, the RCs were dehydrated in an ethanol series, and cleared in methyl salicylate for observation.

Triple staining using fluorescent immunocytochemistry was performed on the RC of male pupae that were in the pharate adult

stage about 24 h before eclosion, collected on ZT 0 under TC 12:12. After fixation with 4% PFA, the RCs were processed for the primary antibody incubation of goat anti-*D. melanogaster*-PER antiserum diluted at 1:1000 with 10% normal donkey serum (NDS) in PBST for 2 days at 4°C. After washing with PBST, the RCs were incubated in 10% NDS in PBST for 1 h at room temperature and kept in the secondary antiserum, donkey anti-goat immunoglobulin conjugated with biotin diluted at 1:200 with 10% NDS in PBST for 1 day at 4°C. After washing with PBST, the RCs were treated with horseradish peroxidase (HRP)-conjugated streptavidin (Invitrogen, California, USA) diluted at 1:100 with PBST for 1 h at room temperature. After washing with PBST, these were kept in Alexa Fluor 488 tyramide (Tyramide Signal Amplification Kit #22, Invitrogen) diluted at 1:100 with PBST containing 0.0015% H_2O_2 for 1 h at room temperature to visualize the enzymatic activity of bound HRP. After washing with PBST, the RCs were incubated in 10% NDS in PBST for 1 h at room temperature and kept in the primary rabbit anti-*D. melanogaster*-adipokinetic hormone (AKH) (Université de Bordeaux, Talence, France, courtesy of Dr. J. A. Veenstra) diluted at 1:500 with 10% NDS in PBST for two days at 4°C. After washing with PBST, the RCs were incubated in 10% NDS in PBST for 1 h at room temperature and kept in the secondary antiserum, tetramethylrhodamine isomer R (TRITC)-conjugated swine anti-rabbit IgG antibody (R0156, Dako, Glostrup, Denmark) diluted at 1:200 with 10% NDS in PBST for one day at 4°C. After washing with PBST, these were treated with 3-μM 4',6-diamino-2-phenylindole (DAPI, Wako Pure Chemical Industries, Osaka, Japan) in PBST for 1 h at room temperature around 26°C. These were dehydrated in an ethanol series, cleared in methyl salicylate, and observed under an epifluorescent microscope (BX50-43FLA-3, Olympus), using a high-pressure mercury burner equipped with NIBA, WIG, and WU filter sets for Alexa Fluor 488, TRITC, and DAPI, respectively. Digital images were processed using NIS-Elements Basic Research 3.0 (NIKON, Tokyo, Japan) and Adobe Photoshop CS5 (Adobe Systems, California, USA).

ELISA

Newly formed pupae reared under TC 12:12 were collected within 1 h from puparium formations. About 3.3 μL hemolymph from each pupa was collected using a 10-μL glass capillary at ZT 0 and 12 from 0 to 9 days after puparium formation, and the last two days of the pharate adults. By morphological features, the pharate adults one and two days before eclosion were easily determined after removal of pupal case. Hemolymph from three pupae of the same age was pooled to make up a 10 μL sample and immediately mixed with 300 μL methanol for

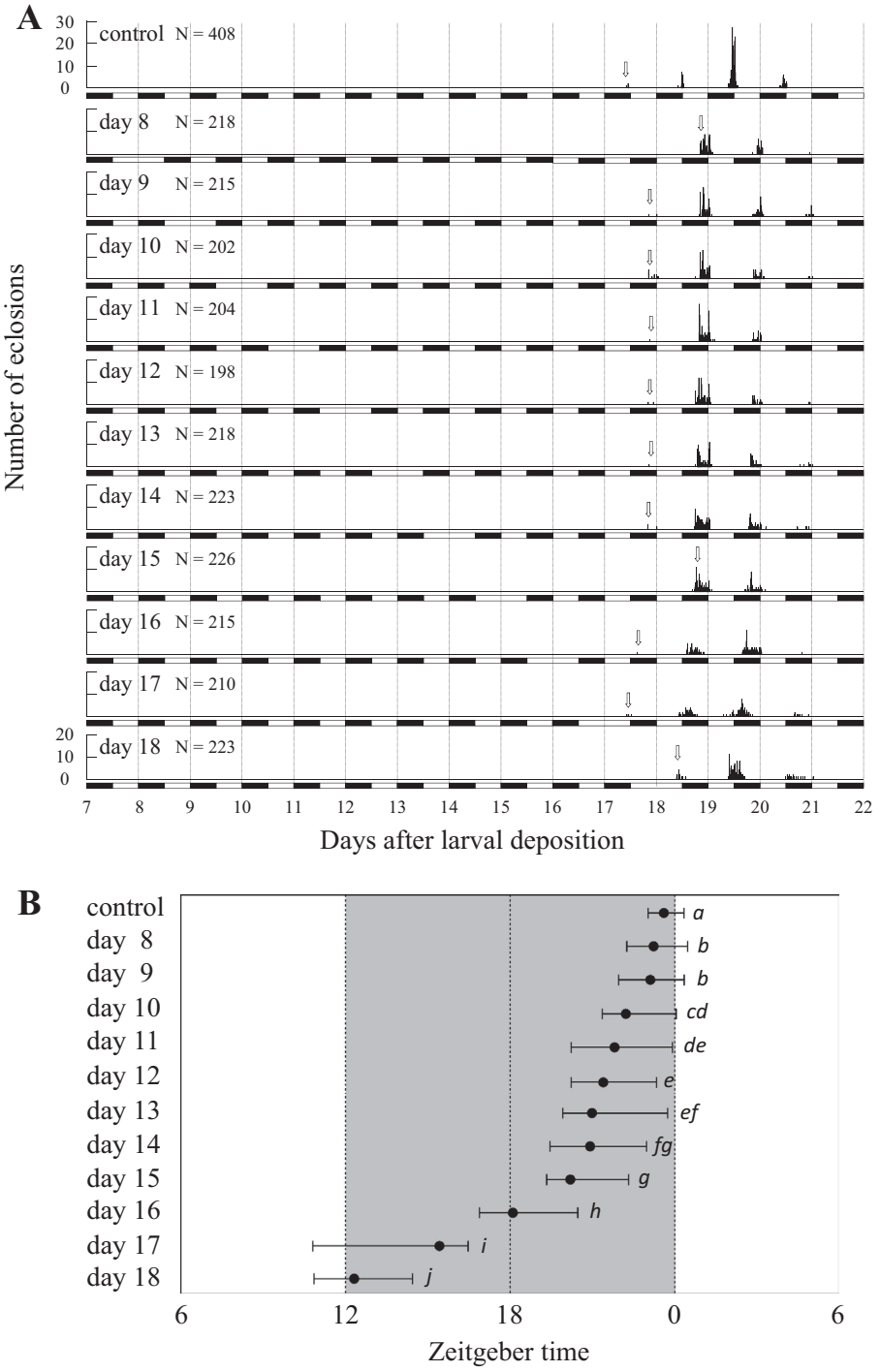

Fig. 1. Effects of a 180°-phase shift of the temperature cycle at different days (day 8–18) during the pupal period on *Sarcophaga crassipalpis* eclosion rhythms in temperature cycles and continuous light. **(A)** Distributions of adult eclosion at 6 min intervals. White and black bars indicate temperature conditions of 28°C and 22°C, respectively. White arrows indicate start of eclosion. **(B)** Plots of medians and interquartile ranges of each peak on the zeitgeber time (ZT) of the temperature cycle. Observed eclosion events were summed on the same ZT among different days. Eclosion plots with different letters show significant differences (Steel-Dwass test, $P < 0.05$). White regions show thermophase and gray regions show cryophase.

extraction. These samples were centrifuged at 12,000 *g* for 5 min at 4°C. The supernatant was collected and lyophilized. Amounts of 20E in the hemolymph were measured by a competitive enzyme-linked immunosorbent assay kit using 20E EIA antiserum and 20E AChE tracer (ACE™ Enzyme Immunoassays, Cayman chemicals, MI) according to the manufacturer's instructions. Plates were read in a plate reader (ARVO™X, PerkinElmer, MA) at 405–420 nm.

Ecdysteroid treatment

20E (Sigma-Aldrich) was dissolved in 10% ethanol to make a series of concentrations at 2, 0.2, 0.02, 0.002, or 0.0002 mg/ml. Only 10 % ethanol was injected in a control group. One μl of 20E (2000, 200, 20, 2, or 0.2 ng/fly) or 10% ethanol was injected into the abdomen with a micro-syringe with a needle (20 mm length, 0.31 mm outside diameter, 0.12 mm inside diameter, Ito Co., Shizuoka, Japan).

Eclosion events were recorded by using a web camera (NET COWBOY DC-NCR13U, Hanwha Japan, Tokyo, Japan). Thirty pupae were aligned in a row with double-sided adhesive tape beneath a transparent lid of a plastic box (25.5 cm length, 18.5 cm width, 9.0 cm depth). One box contained eight rows of 30 pupae (240 pupae). The box was half filled with water, into which emerged adults fell. Pupal rows were photographed every 6 min by the camera set directly above the plastic box for six days after the first peak of eclosion was observed.

RESULTS

Effects of temperature cycles on eclosion rhythm

When flies were kept under TC 12:12 from the larval period, eclosion occurred between days 18 and 20 and a clear population rhythm was observed with eclosion events around the start of thermophase (control in Fig. 1A). An eclosion gate was observed with a peak at ZT 23.5 (median, n = 408) just before the thermophase started, and the interquartile range of eclosion events was 1.1 h (ZT 23.1–ZT 0.2) (control in Fig. 1B). This rhythm free-ran with a free-running period of 24.1 ± 0.4 h (n = 4), when pupae were transferred from TC 12:12 to constant conditions of 28°C at ZT 0 on day 17 (Supplementary Figure S1A_1a, A_2a, B_1a, B_2a). To examine responsiveness of the rhythm to a phase shift of temperature cycles during the pupal period, a 180°-phase shift was given by a skip of either 12 h (180°) cryophase from day 8, when the majority of puparium formation occurred in TC cycles, to day 18 just before eclosion (Fig. 1A). The later the

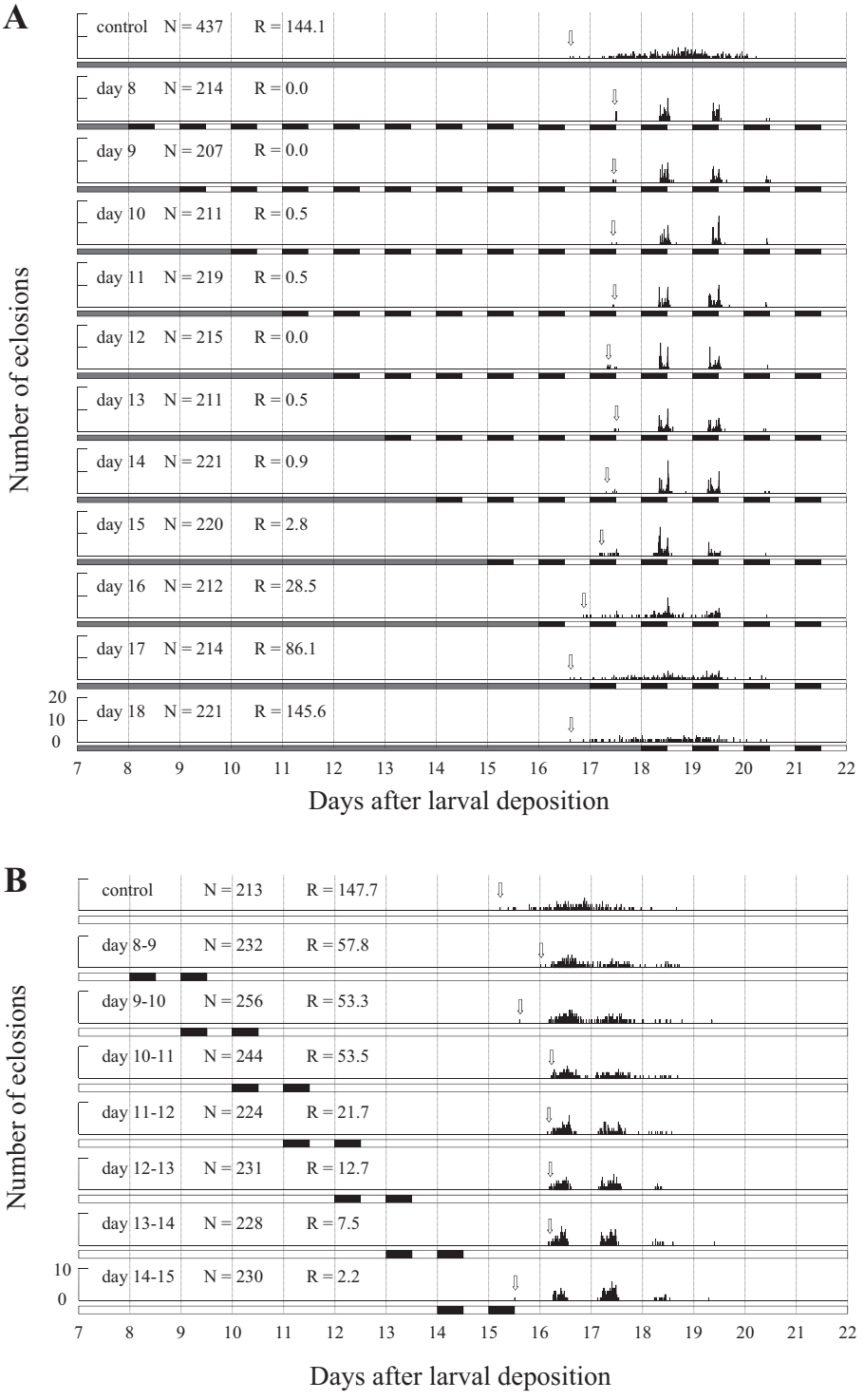

Fig. 2. Effects of temperature cycles on *Sarcophaga crassipalpis* eclosion rhythms in continuous light. Distributions of adult eclosion are shown at 6 min intervals. **(A)** Pupae were transferred from a constant temperature of 25°C to temperature cycles at different pupal stages. **(B)** Pupae under a constant temperature of 28°C were provided with two temperature cycles at different pupal stages. White bar, 28°C; gray bar, 25°C; black bar, 22°C. White arrows indicate start of eclosion.

phase shift, the farther the eclosion peak from the start of thermophase (Fig. 1B). The shift on day 10–15 caused gradual advances of the eclosion peaks, but most eclosion events were still found in the latter half of cryophase. By the shift on day 16–18, eclosion occurred in the middle or at the beginning of cryophase which was apart from eclosion phase in the control group and eclosion peak became weakened, indicating that the rhythm was not able to entrain to a shifted TC cycle (Fig. 1). We considered rhythms in day 8–15 shifted groups entrained mostly to zeitgeber, and it seemed that at least 3 TC cycles were necessary for a 180° phase shift.

Next, the numbers of TC cycles and the sensitive stage for rhythm occurrence were examined. When flies were kept under constant conditions of 25°C for several generations, eclosion events occurred in an arrhythmic manner (control in Fig. 2A). To examine the generation of eclosion rhythms by TC cycles in the pupal period, TC cycles were given on different days in constant conditions. Under the constant conditions of 25°C, puparium formation occurred in the majority on day 7. When pupae were transferred to TC cycles on day 8–15, a clear eclosion rhythm was observed with eclosion events around the start of thermophase (Fig. 2A). When pupae were transferred to TC cycles on day 16, less clear rhythms appeared and a peak-like eclosion profile was observed after 2 TC cycles (Fig 2A). By the transfer on day 17, rhythmicity was detected according to R value but it was very weak. By the transfer on day 18, eclosion occurred in an arrhythmic manner. Clear rhythmicity was observed when two or more TC cycles were given before eclosion (Fig. 2A).

In the next experiment, 2 TC cycles were given at a different stage during day 8–15 in constant conditions of 28°C and the population rhythm was examined (Fig. 2B). Two TCs in any stage caused rhythmic eclosion (R < 60) and those on day 11–12 or later caused highly rhythmic eclosion (R < 30) (Fig. 2B). The clearest rhythm was observed by the last 2 TCs in the pupal period just before eclosion, according to R values. The results indicated that the circadian clock is able to entrain to TC cycles or to start rhythms 2 days before eclosion. This suggests that circadian clocks release timing signals for eclosion onset later than 48 h before eclosion.

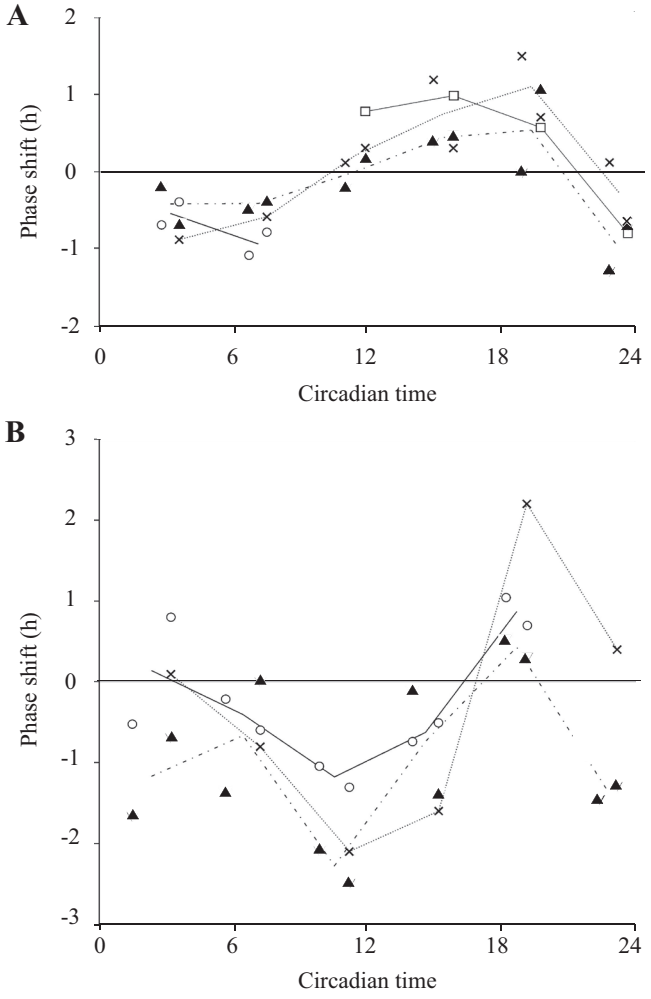

Fig. 3. Phase response curves for *Sarcophaga crassipalpis* eclosion rhythms to a 4-h low-temperature pulse of 22°C **(A)** and a 1-h low-temperature pulse of 4°C **(B)** 1 day before eclosion (circle), two days before eclosion (cross), three days before eclosion (triangle), and four days before eclosion (square). The phase shift values, where positive values are phase advances and negative values are phase delays, in circadian time were plotted as a function of the phase where the pulses were started. CT, circadian time.

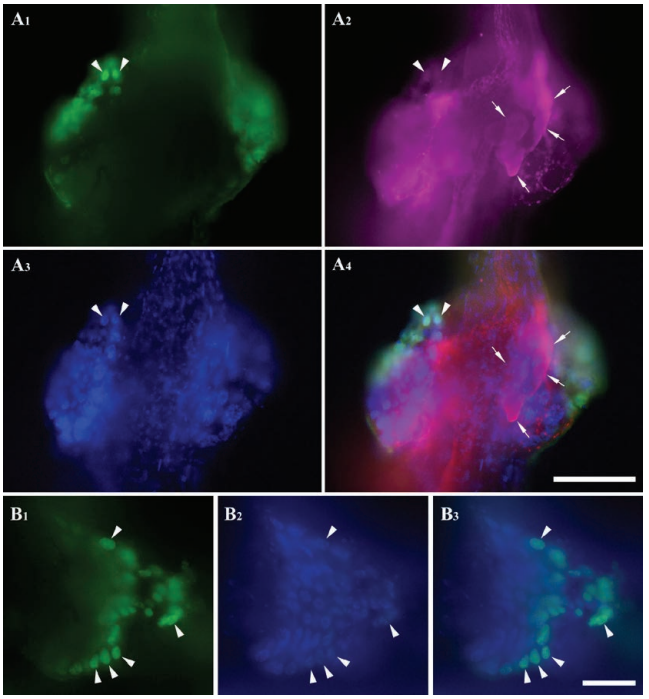

Fig. 4. Fluorescent micrographs of the triple labeling of a retrocerebral complex at zeitgeber time 0, about 24 h before eclosion, with anti-PERIOD antisera (green), DAPI (blue), and anti-AKH antisera (magenta) in male *Sarcophaga crassipalpis*. A whole view **(A)** and a region of the prothoracic gland **(B)** of the retrocerebral complex are shown. PERIOD-immunoreactive cells (arrowheads) and AKH-immunoreactive cells (arrows) were found in the different regions **(A)**. In merged images (A_4 and B_3), co-localization of PER-immunoreactivity and DAPI staining was observed. Arrowheads indicate exactly the same positions each in A_1–A_4 and in B_1–B_3. Scale bar, 100 μm in **(A)**; 50 μm in **(B)**.

Effects of a low temperature pulse on eclosion rhythm

The latest phase at which eclosion timing was affected by a zeitgeber was examined using a low-temperature pulse. When pupae were transferred to constant conditions of 28°C from TC 12:12 at ZT 0 on day 17, eclosion rhythm free-ran (Supplementary Figure S1A_1a, A_2a, B_1a, B_2a). When a low temperature pulse of 22°C for 4 h was given at different phases, eclosion peaks delayed at around CT 2, 6, and 24, and phase advances were observed at around CT 14 and 18 (Fig. 3A). Phase-shift amounts at similar CT were not largely different among days on which the pulse was given. When the latest 4 h-pulse was given at CT 7.1 which was 16.9 h in CT before eclosion on the last day of pupae, a phase delay by 0.9 h in CT was observed (Fig. 3A). To examine responsiveness of the rhythm to zeitgeber at later stages, a shorter pulse of 1 h at 4°C was used (Supplementary Figure S1B). Peak values of the phase shift became larger than those with a 4-h pulse at 22°C, and a clear delay at around CT 10 and advance around CT 18 were observed (Fig. 3B). A 1-h pulse given at CT 18.6 on the day before eclosion, which was 5.4 h in CT before eclosion, caused a phase advance of the eclosion peak by 0.9 h in CT (Fig. 3B).

PER-immunoreactive cells in the PG and 20E titers in the hemolymph

PER-immunoreactive cells were examined in the ring gland or metamorphosing RC in the last 2 days of pupae. Triple labeling of the RC with PER immunocytochemistry, AKH immunocytochemistry, and DAPI staining was performed about 24 h before eclosion, ZT 0 (Fig. 4). AKH-immunoreactive cells were found in the medial and proximal part of the RC. As AKH has been produced in the corpus cardiacum (CC) of *D. melanogaster* and the blow fly *Protophormia terraenovae* (Gade et al., 1990; Isabel et al., 2005), AKH-immunoreactive regions are considered the CC. Cells with colocalized staining of PER-immunoreactivity and DAPI were observed bilaterally in the PG regions just lateral to the CC (Fig. 4A). Colocalization with PER-immunocytochemistry and DAPI indicates that the nuclei were immunopositive to PER (Fig. 4B).

Occurrence of PER immunoreactive nuclei in the PG was examined every 6 h during the last two days before eclosion under TC 12:12. Stained intensity changed during a day. PER immunoreactivity in the nuclei was classified into 3 patterns. In the strong-staining pattern many nuclei were distinctively labeled with anti-PER antiserum (Fig. 5A_1), whereas in other preparations no staining was observed in the nuclei but weakly cytoplasm was stained (Fig. 5A_2). In the weak-staining pattern, the staining intensity was intermediate (data not shown). The proportion of individuals with strong or weak PER staining in the PG nuclei was more than 70% about 48 h before eclosion (ZT 0), but it gradually decreased (Fig. 5B). The proportion again increased around 24 h before eclosion (ZT 0). At around ZT 12 and 18, PER immunoreactivity was not observed in all individuals and it appeared again just after emergence (ZT 0). No PER immunoreactivity was observed in the RC 6 h after eclosion (n = 6, data not shown). Thus, daily variation of PER-immunoreactivity was observed. At about 24 h before eclosion (ZT 0), these cells exhibited no immunoreactivity when the primary antiserum was absorbed with the blocking PER-peptide (n = 5), indicating that the staining

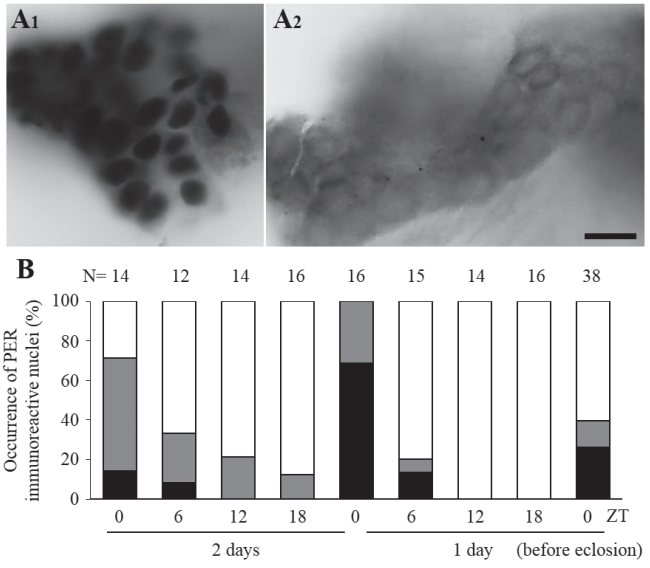

Fig. 5. Daily changes in PERIOD immunoreactivity in the prothoracic gland of male *Sarcophaga crassipalpis*. **(A)** Representative photomicrographs of PERIOD-immunoreactive cells in the retrocerebral complexes of whole mount preparations 24 h (A_1) and 12 h (A_2) before eclosion. About 12 h before eclosion, nuclei were completely immunonegative, but slightly cytoplasm was labelled (A_2). Scale bar, 10 μm. **(B)** Occurrence of PER-immunoreactive nuclei in the retrocerebral complexes under temperature cycles. Black columns show individuals with strong PERIOD immunostaining in the nuclei (A_1). Gray and white columns show weakly staining and lack of staining in the nuclei (A_2), respectively. Thermophase starts at zeitgeber time (ZT) 0 and finishes at ZT 12.

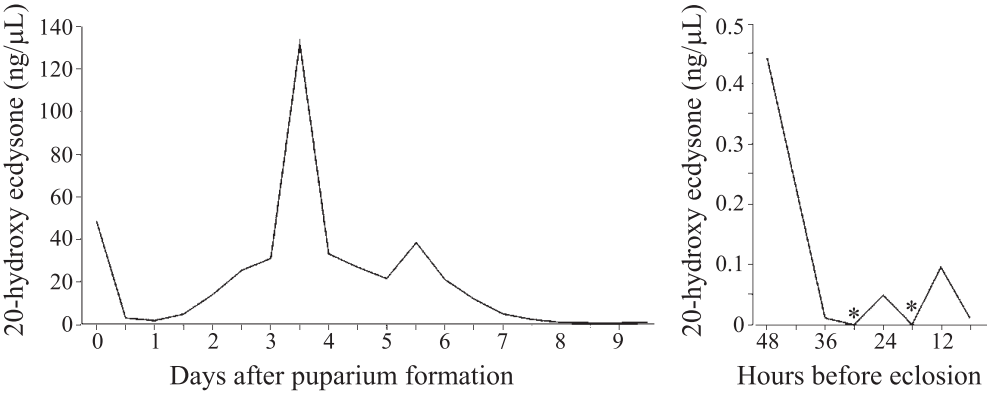

Fig. 6. Hemolymph titers of ecdysteroids under temperature cycles during pupal periods of *Sarcophaga crassipalpis*. The pharate adults one and two days before eclosion were easily determined by morphological features, after removal of pupal case. Hemolymph from three pupae of the same age was pooled to make up a 10 μL sample at each point. Asterisks, undetected.

was PER-specific.

To consider the possibility that cycling of PER-immunoreactivity in the PG may control production or release of ecdysteroids in the last two days of pupae, hemolymph titers of 20E were examined under TC cycles. In the pupal period a large 20E peak was observed 3.5 days and a smaller one 5.5 days after puparium formation (Fig. 6). After the second small peak, 20E titers gradually decreased. It appeared that no daily changes occurred in the titers. Throughout the last two days before eclosion, 20E titers were very low and undetected at some points.

Effects of 20E injection on the eclosion time

20E injection was performed at ZT 4, 8, 12, or 16 under temperature cycles one day before the first eclosion peak to examine effects on eclosion time (Fig. 7). Eclosion times were not different between intact and control groups at all injection times. A series of concentrations from 0.2–2000 ng 20E was injected at ZT 4 and 8 (Fig. 7A, B). By the 2000 ng injection all pupae died at ZT 4 injection (n = 10) and 90% died at ZT 8 (n = 10). By ZT 4 injection, 0.2, 2 or 20 ng 20E caused a significant delay of the eclosion time in the first eclosion group compared with the intact or control group (Fig. 7A). Although the second eclosion population was very small, no delay in eclosion time was observed. When 200 ng 20E was injected at ZT 4, the survival rate was only 20% (n = 30) and surviving pupae came out at a much later phase. By ZT 8 injection, 20 or 200 ng 20E caused a significant delay in the first population, but the delay was not large when compared with 20E injection at ZT 4 (Fig. 7A, B). The survival rate in the 200 ng 20E injection at ZT 8 was 70% (n = 30). At ZT 12 and 16, only 2 and 20 ng 20E injections were made, and neither had an effect on the eclosion times (Fig. 7CD). In 20 ng or lower 20E injections, pupae survived at a range of 62.5–90% (n = 30–40). The results showed that the effects of 20E were time-dependent.

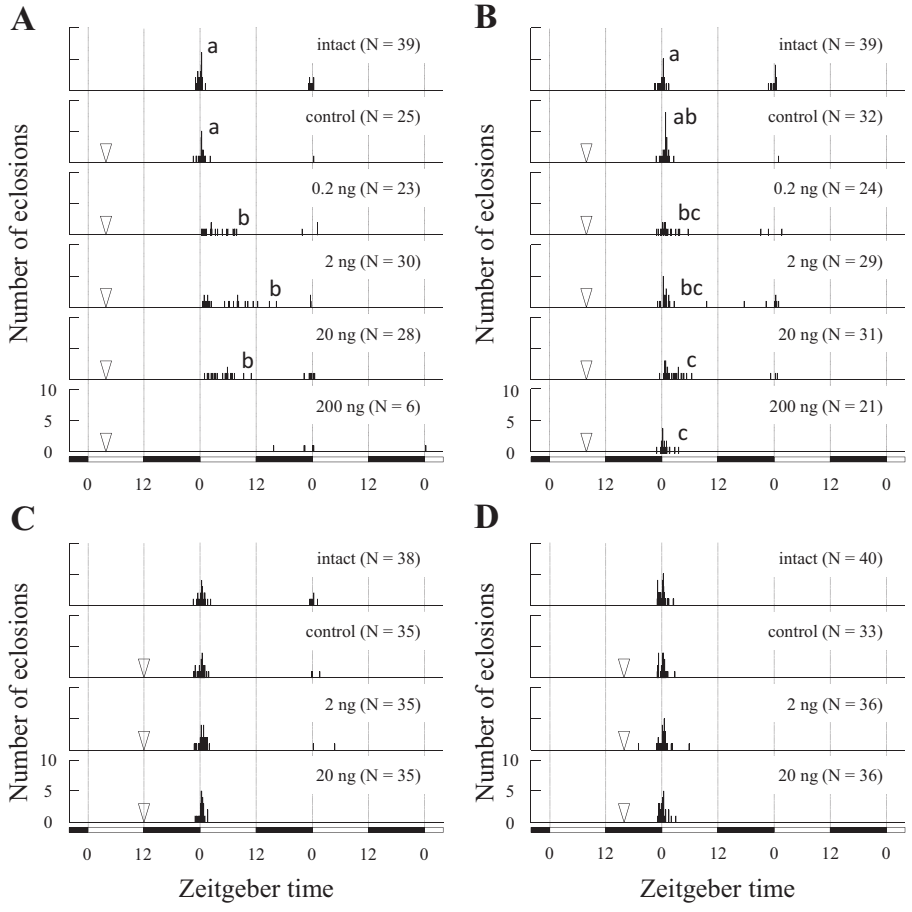

Fig. 7. Effects of 20-hydroxyecdysone (20E) injections on the eclosion time of *Sarcophaga crassipalpis* in temperature cycles. White and black bars indicate temperature conditions of 28°C and 22°C, respectively. The number of eclosion events in a 6-min bin is plotted against zeitgeber time (ZT). Inverted triangles show the injection time. Different letters on the plot show significant differences in eclosion time in the first population (Steel-Dwass test, $P < 0.01$). Boundary between the first and second peak was arbitrarily set at ZT 18. ZT 4 **(A)** and 8 **(B)**, 20E delayed the eclosion time. No significant effects were observed by 20E at ZT 12 **(C)** and 16 **(D)**. As a control, 10% ethanol was injected.

DISCUSSION

Signal timing of circadian clocks for eclosion onset

We used temperature as a zeitgeber signal because *S. argyrostoma* pupae respond to temperature but not light to entrain eclosion rhythms (Saunders, 1976). *Sarcophaga crassipalpis* pupae were also not responsive to light for entrainment of the eclosion rhythm (data not shown). Pupae of the onion fly *Delia antiqua* (Diptera: Anthomyiidae) are responsive to both light and temperature, but the latter is more important to determine eclosion phases (Watari, 2002). Since both onion flies and fresh flies pupate underground, where light does not penetrate, temperature rather than light is a suitable zeitgeber for the eclosion rhythm during the pupal stage (Miyazaki et al., 2011).

It has been demonstrated that the eclosion gate is governed by the circadian clock (Saunders, 2002). To clarify the circadian gating mechanism it is important to know when the circadian clock submits output signals for eclosion onset, because gating is a temporal coordination by the circadian clock and hormonal events for developmental processes. However, this timing has not been revealed in any species. A 180°-phase shift and transfer from constant conditions to TC cycles showed that eclosion rhythm was responsive to a new TC cycle during the last 2–3 days before eclosion to change eclosion time. This indicates that during the period the circadian clock controlling the eclosion gate receives zeitgeber signals to change the phase or start driving, and submits signals to set or change eclo-

sion time.

To identify the latest moment when the circadian clock shifts the phase for eclosion time, PRCs were drawn using a temperature pulse. The width of eclosion peaks is 1.1 h (the interquartile range) under zeitgeber cycles in *S. crassipalpis*, which is much narrower than that observed in *D. melanogaster* (11.89 ± 0.15 h) and in *D. pseudoobscura* (several hours) (Pittendrigh, 1954; Kannan et al., 2012). Sharp eclosion peaks in *S. crassipalpis* are advantageous to differentiate eclosion peaks with small phase differences. In *D. pseudoobscura* PRCs for eclosion rhythm have been drawn using high- or low-temperature pulses of 12 h administrated to pupae in 4–7 days before eclosion, and the amplitude of the phase shift was slightly larger from a low-temperature pulse than a high-temperature pulse (Zimmerman et al., 1968). Subsequently, we chose a low-temperature pulse to examine the phase response in the last pupal day. A low-temperature pulse on the day just before the first peak of eclosion caused phase delays and advances in a phase-dependent manner, and PRCs for *S. crassipalpis* eclosion rhythm in the present study are similar to *D. pseudoobscura*, although the pulse length and developmental stages (pupal days) at which the zeitgeber was given were different. In *D. pseudoobscura*, a 3-h low-temperature pulse of 10°C against 20°C administered 4–7 days before eclosion causes phase advances at around CT 14 to 18, and phase delays at around CT 22 to 12 with the maximum of the phase shifts of about 2 h (Chandrashekaran, 1974). In *S. crassipalpis*, a low-temperature pulse of 1 or 4 h applied at around CT 18 also induced a phase advance. A 1-h low-temperature pulse of 4°C at CT 18.6 of the last pupal day led to phase advancing by 0.9 h. This phase-shift was the latest one we could examine. In the current study, we set CT 0 or CT 24 for the eclosion peak, meaning that CT 18.6 was 5.4 h before eclosion. The 0.9 h phase advancement led CT 18.6 to CT 19.5, which was only 4.5 h before eclosion. The interval from the zeitgeber input to eclosion behavior was 4.5 h, indicating that eclosion time was determined, or could be changed, by cues submitted from the circadian clock 4.5 h before the behavioral onset or even at a later stage. When phase shift amounts are compared between 4 h pulse of 22°C and 1 h of 4°C, the latter causes a larger effect. In phase response curves using light pulses, longer period or stronger light intensity causes larger phase shift in the cockroach *Leucophaea maderae* (Wiedenmann, 1977). Phase response curves in our study suggest that also lower temperature causes a larger phase shift of the circadian clock driving the eclosion rhythm even by a shorter period of the pulse in *S. crassipalpis*.

Pittendrigh (1960, 1965) examined effects of a light pulse just before eclosion on the eclosion timing in *D. pseudoobscura*, in which eclosion peak occurred at CT 3.5 without the light pulse. A 15-min light pulse on the day before eclosion caused significant phase delays but not advances. The pulse at CT 18 was the latest point examined in *D. pseudoobscura* and caused a pronounced phase delay (Pittendrigh, 1965). CT 18 was 9.5 h before eclosion at CT 3.5, meaning that a duration from zeitgeber input to eclosion behavioral onset is 9.5 h. In the present study the duration was shortened to 4.5 h.

Roles of the PG and of 20E in eclosion time

It has been demonstrated in *D. melanogaster* that the PG in pupae contains circadian clock cells, in which PER protein in the nuclei oscillates in vivo, and a *per-luciferase* reporter gene has revealed circadian rhythms of bioluminescence in vitro in light-dark cycles and constant darkness (Emery et al., 1997; Morioka et al., 2012). In *S. crassipalpis*, strong PER immunostaining was also found in nuclei of the PG cells and the occurrence of PER positive cells oscillated under temperature cycles with the peak around ZT 0 at the start of thermophase. ZTs with PER positive phases are similar between *D. melanogaster* and *S. crassipalpis*, although zeitgeber cues are different between the two species (Emery et al., 1997). PER immunostaining in the PG nuclei was very distinct around the onset of thermophase and showed a large contrast to completely negative profiles during the cryophase, suggesting that strong daily oscillation occurs in these cells during the last two days before eclosion. Although we have not examined PER staining under constant conditions in *S. crassipalpis*, regarding results from *Drosophila* it is probable that PER occurrence in the PG also oscillates under constant conditions in *S. crassipalpis,* and PG cells play an important role in circadian rhythmic events. Myers et al. (2003) have shown that overexpression of a circadian clock gene *tim* in the PG affects circadian eclosion rhythm, and an interaction between the PG and the central circadian clock system in the brain is important for driving the eclosion rhythm. These results suggest in *D. melanogaster* that the PG contains peripheral clocks giving an output signal for eclosion timing in accordance with its own clock, and central clocks are important for the peripheral clock to submit proper timing signal for eclosion. In our experiments central clock and peripheral clock are indistinguishable but the PG may function as peripheral clock as in *D. melanogaster*.

The PG primarily produces and releases ecdysteroids (Nijhout, 1994). We examined the possibility that ecdysteroids are released from the PG just before eclosion. However, hemolymph titers of 20E were quite low in the last two days before eclosion. There were no increments observed in *S. crassipalpis*, and no circadian oscillation in 20E titers was observed during the pupal period. In a congeneric species *S. bullata*, ecdysteroid titers also decreased invariably 35 h before eclosion and no circadian change was observed (Wentworth et al., 1981). Therefore, it is unlikely that 20E represents the eclosion timing signal from the circadian clock cells in *Sarcophaga*. However, there might be a very sharp and small peak of ecdysteroids that was not detected by our method. To test this possibility, we injected ecdysteroids and examined their effects on eclosion time. If 20E plays a role as a timing signal submitted by the circadian clock, 20E injection in the last pupal day should advance eclosion time. However, it caused a delay of eclosion in time dependent manner. The pupae delayed eclosion time when 20E was injected at ZT 4 (about 20 h before eclosion) and ZT 8 (about 16 h before eclosion) but no significant effects were observed at ZT 12 (about 12 h before eclosion) or ZT 16 (about 8 h before eclosion). This suggests that 20E is not a triggering signal of eclosion behavior.

Effects of ecdysteroid injection on the eclosion time have been examined in the mealworm beetle, *Tenebrio*

molitor (Coleoptera: Tenebrionidae) and *M. sexta*. In these species as well, ecdysteroid injections affected the eclosion time in a time-dependent manner: earlier injection inhibited or delayed eclosion, but the injection at the last moment before eclosion did not have significant effects (Sláma, 1980; Truman et al., 1983). The results in *S. crassipalpis* concurred with these species. The injections at ZT 4 and 8 caused delays only in the first peak, but did not seem to cause effects on the second one (Fig. 7A, B). Although it could be that the second peak was too small to evaluate the effects of 20E, we think that pupae in the second eclosion peak were at an insensitive stage to 20E, when they were injected with 20E at ZT 4–8. That is, *S. crassipalpis* seems to have a narrow window which is sensitive to 20E 20–16 h before eclosion. Sláma (1980) suggests that ecdysteroids have an effect to inhibit ecdysis behavior thereby avoiding premature ecdysis before cuticle formation and apolysis. During the premature period, the pharate adults are sensitive to 20E, and in the mature stage, after completion of cuticle formation and apolysis, they become insensitive. 20E levels may be an indicator to avoid premature eclosion in *S. crassipalpis*. When 20E is low at the sensitive stage pupae eclose in the proper gate, whereas if 20E is still high at that stage pupae delay eclosion until developmental completion.

According to Pittendrigh (1965), when the gate signals come from the circadian clock at a certain phase and development has not been completed, eclosion is skipped to the next gate. However, in *S. crassipalpis* a delay of eclosion by hours, not a skip of days, were observed by 20E injection at the sensitive stage (Fig. 7A, B). In *M. sexta* also, low doses of 20E 28 h before eclosion caused pushing eclosion events away from the normal gate by several hours (Truman et al., 1983). To explain reasons why 20E causes a delay of hours not a jump to the next day, we think that there is a mechanism to set the eclosion day, presumably by some hormonal event. Day setting occurs probably just before the 20 E sensitive stage, which occurs 20–16 h before eclosion, in *S. crassipalpis*. If 20E is not detected after determination of the eclosion day, eclosion occurs in the gate set by the clock. If it is detected after the day setting, eclosion day cannot be changed but its time was delayed by several hours until complete maturation. Because delayed eclosion events by 20E injection occurred in a dispersed manner, not in a peak, 20E injection did not appear to cause a phase shift of the circadian clock, but rather to delay the final events of development. Probably the clock submitted a timing signal as usual but pupa was not ready to accept the signal to eclose in the gate, resulting a delay of eclosion by several hours. We think that earlier injection of 20E may cause a jump of eclosion to a later gate. Hormonal events control developmental rates including determination of an eclosion day, and the circadian clock controls an eclosion time of the day. Consequently hormonal events and circadian clocks act in concert to determine eclosion gate (Di Cara and King-Jones, 2013).

In the hemolymph 20E declined before the 20E sensitive period in *S. crassipalpis*. A decline in ecdysteroids towards the end of the molt is essential for molting process (Sláma, 1980). In *Manduca sexta*, an increase of ETH secreted from grand cells on the trachea requires a drop of the ecdysteroid titer prior to eclosion (Kingan et al., 1997). ETH induces secretion of EH from central ventromedial neurons in the brain (Clark et al., 2004), and EH acts in a positive-feedback loop to lead further release of ETH (Ewer et al., 1997). A decline of 20E titers is also necessary for an increment in the responsiveness to EH and its titer in the hemolymph (Truman et al., 1983). Subsequently EH triggers release of crustacean cardioactive peptide, which turns on the ecdysis motor program (Gammie and Truman, 1999; Park et al., 2003). Although neither EH nor ETH have been identified, probably a similar peptidergic cascade functions to control eclosion behavior in *S. crassipalpis* and a low level of ecdysteroids 20 h before eclosion or earlier is necessary for their function.

Considering the fact that PG cells produce ecdysteroids, it remains unsolved why the PERIOD oscillation is observed in PG at the last moment of pupae. It may have no functional roles in the eclosion gate, or may control the submission of unknown products to set the gate. Ewer (2007) suggested that PG has a currently unknown function in eclosion, that is unrelated to ecdysteroid production, but required for circadian rhythms. Our results also support this idea. Production of substances other than ecdysteroids has been suggested in adults of the desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae) (Boerjan et al., 2012). Therefore, to clarify the role of PG in the final stage of pupae in the eclosion gate, PG ablation and biochemical analysis at this stage is necessary in future studies. Phase responses to low temperature pulses suggest that the circadian clock can change the eclosion time even 4.5 h before eclosion. The PG clock probably submits a clock-timing signal different from 20E for eclosion onset just before eclosion.

ACKNOWLEDGMENTS

We thank Dr. Kouji Yasuyama from Kawasaki Medical School for discussions on this project, and Dr. J. A. Veenstra from Université de Bordeaux for providing the antisera against AKH. This work was supported by a Grant-in Aid for Scientific Research (C), 22570076 by the Japan Society for Promotion of Science, and the Narishige Zoological Science Award in 2014.

COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

MY and SS designed and wrote the paper. KN performed ELISA and part of immunocytochemistry experiment, and MY performed the other experiments.

SUPPLEMENTARY MATERIALS

A supplementary material for this article is available online (URL: http://www.bioone.org/doi/suppl/10.2108/zs160153).

Supplementary Figure S1. Effects of a low temperature pulse on the phase of *Sarcophaga crassipalpis* eclosion rhythms.

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(Received September 15, 2016 / Accepted November 25, 2016)