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ORIGINAL RESEARCH PAPER

No evidence for the involvement of juvenile hormone III and 20-hydroxyecdysone in maternal decisions for embryonic diapause and diapause entry in the band-legged ground cricket *Dianemobius nigrofasciatus* (Orthoptera: Trigonidiidae)

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Abstract
Diapause is a programmed stage-specific arrest or delay in reproduction or development and is commonly used to circumvent an adverse season. Some insect species exhibit maternal regulation of diapause, wherein environmental cues are perceived by the mother and subsequently determine the developmental fate of the offspring. Although maternal regulation of diapause is widespread, its endocrinological mechanisms remain largely unknown. In the band-legged ground cricket *Dianemobius nigrofasciatus* (Orthoptera: Trigonidiidae), embryonic diapause is maternally determined. Adult females under long-day conditions lay eggs that develop into nymphs without interruption, whereas those under short-day conditions lay diapause-destined eggs that arrest their development and enter diapause at a very early embryonic stage, the cellular blastoderm. How development is arrested at an early stage is a key area of interest. We hypothesized that juvenile hormone III (JH III) and 20-hydroxyecdysone (20E), the major insect hormones that regulate a wide variety of physiological processes, are involved not only in maternal decisions, but also in diapause entry in *D. nigrofasciatus*. The results showed that the hemolymph concentrations of JH III and 20E in adult females were lower under short-day conditions; however, the application of JH III and 20E to the mothers did not affect the diapause incidence of offspring. No differences were observed in the amounts of 20E between non-diapause and diapause-destined eggs, and JH III was not detected in these eggs. Thus, we found no evidence for the involvement of JH III and 20E in maternal decisions for embryonic diapause and diapause entry in *D. nigrofasciatus*.

Keywords Ecdysteroid · Hormonal regulation · Juvenile hormone · Maternal effect · Photoperiodism · Seasonal adaptation

Introduction

Seasonal changes in the environment impose physiological challenges on many organisms including insects. Many insect species enter diapause to overcome seasons that are unsuitable for reproduction or development. Diapause is a programmed stage-specific arrest or delay in reproduction or development that occurs before an upcoming adverse season (Denlinger 2022; Goto 2022). In many species, individuals that receive token stimuli enter diapause. However, in some cases, a fascinating route for programming diapause involves letting the mother decide. The mother receives environmental cues and transfers this information to her progeny. This transgenerational effect is referred to as the maternal effect (Mousseau and Fox 1998). Maternal programming of diapause is particularly valuable for embryonic diapause, a situation in which an individual has limited access to or time to evaluate seasonal conditions on its own. Although this fascinating route has attracted the attention of many researchers, its underlying physiological mechanisms remain unclear.

An iconic species showing maternal programming of diapause is the silk moth *Bombyx mori* (Fujiwara et al. 2006; Sato et al. 2014; Tsuchiya et al. 2021; Chen et al. 2022). When the mother is at the embryonic stage and receives long days at a higher temperature or when the mother is at the larval stage and receives short days at a lower temperature, the mother releases diapause hormone

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(DH), a 24aa neuropeptide belonging to the FXPRL-amide peptide family, from the neurosecretory cells in the suboesophageal ganglion. DH acts on the ovaries to alter carbohydrate metabolism and boost sorbitol levels in the egg yolk (Yamashita 1996; Sato et al. 1998). Horie et al. (2000) demonstrated that the presence of sorbitol in the yolk causes developmental arrest during diapause. Although the role of DH in embryonic diapause induction has also been reported in the spotted tussock moth *Orgyia thyellina* (Uehara et al. 2011) and the locust *Locusta migratoria* (Hao et al. 2019), attempts to induce diapause with DH in most other species have failed (Denlinger 2022).

Insect hormones that regulate a wide range of physiological and developmental processes include juvenile hormones (JHs) and ecdysteroids (Dubrovsky 2005; Jindra et al. 2013). JH is an acyclic sesquiterpenoid that is synthesized at and released from the corpora allata (CA) (Bellés et al. 2005; Li et al. 2019). Although several molecular species of JHs have been reported, many insects use JH III as the innate JH (Daimon and Shinoda 2013). Recently, JH III was reported to be the maternal factor regulating offspring developmental trajectory (larval diapause) in the jewel wasp *Nasonia vitripennis* (Mukai et al. 2022). Under long-day conditions, the mother increases the hemolymph concentration of JH III and produces eggs destined to develop into pupae without interruption. However, under short-day conditions, the hemolymph JH III concentration decreases, and eggs destined to enter larval diapause are produced. Silencing of a JH III biosynthetic enzyme and topical application of JH III revealed a causal relationship between JH III and maternal decisions (Mukai et al. 2022). It is still unknown how JH III regulates the developmental trajectory of the offspring. The mosquito *Aedes albopictus* also exhibits maternal induction of diapause. In this case, JH is involved in diapause entry (Batz et al. 2019). Maternal factors in this species are still unknown.

Ecdysteroids are a class of steroid hormones that are produced from dietary sterols. A series of reduction–oxidation reactions produces ecdysone. Ecdysone is a relatively inactive prohormone that is converted into the much more active form, 20-hydroxyecdysone (20E), by the fat body and epidermal cells, which regulates development, molting, and metamorphosis (Pan et al. 2021). In two locust species, *Chortoicetes terminifera* and *L. migratoria*, which maternally induce embryonic diapause, adult females transmit a larger amount of ecdysteroids to non-diapause-destined eggs than to diapause-destined eggs (Gregg et al. 1987; Tawfik et al. 2002). Diapause of *L. migratoria* eggs can be terminated when they are immersed in 20E solution for 24 h (Kidokoro et al. 2006). These results suggest that the mother regulates embryonic diapause by varying the amount of 20E in eggs. The involvement of ecdysteroids in embryonic diapause has also been proposed in the gypsy moth *Lymantria dispar* (Lee and Denlinger 1996, 1997; Lee et al. 2002).

The band-legged ground cricket, *Dianemobius nigrofasciatus*, regulates embryonic diapause in offspring by photoperiods experienced by the mother. Adult females reared under long-day conditions lay eggs that develop into nymphs without interruption (non-diapause), whereas those reared under short-day conditions lay diapause-destined eggs (Masaki 1972; Shiga and Numata 1997). This species is also unique because eggs destined to enter diapause arrest their development at the cellular blastoderm stage between 40 and 56 h after egg laying (Tanigawa et al. 2009). At this stage, no organs or distinct tissues are formed. This is one of the earliest stages of embryonic diapause in insects. How development is arrested at an early stage is a key area of interest. Although the suppression of cell cycle regulators, small silencing RNA, and segment patterning gene expression is assumed to play a pivotal role in diapause entry (Shimizu et al. 2018), the causal factors regulating embryonic diapause remain unknown.

In this study, we hypothesized that JH III, 20E, or both are involved not only in maternal decisions, but also in diapause entry in *D. nigrofasciatus*. To determine whether they were involved in the maternal decision, we quantified the hemolymph JH III and 20E in adult females reared under long-day and short-day conditions. We applied JH III, 20E, or both to adult females to determine whether these hormones affected maternal decisions. In *N. vitripennis*, the topical application of small amounts of JH III to adult females immediately alter their maternal decision from diapause producers to non-diapause producers (Mukai et al. 2022). Although no chemical application has been conducted in *D. nigrofasciatus*, the application of JH III and/or 20E would also immediately affect the maternal decisions in this species if these hormones are involved. We further measured the amounts of JH III and 20E in diapause-destined and non-diapause-destined eggs to determine the possible involvement of these hormones in embryonic diapause. Contrary to our prediction, we found no evidence for the involvement of JH III and 20E in maternal decisions for embryonic diapause and diapause entry in *D. nigrofasciatus*.

Materials and methods

Insects

Adults and nymphs of *D. nigrofasciatus* were collected in a grassy field on the campus of Osaka City University (34° 35′ N, 135° 30′ E) and the bank of the Yamato-gawa River (34° 35′ N, 135° 30′ E), Japan, from June to November 2021 and 2022. Approximately, 40–60 individuals were reared in a plastic case (29.5 cm width, 19.0 cm depth,

and 17.0 cm height) under long-day (LD) conditions (16-h light:8-h dark, 25.0 ± 1.0 °C) with a piece of moist cotton as a water source and oviposition site. They were fed an artificial insect diet (Oriental Yeast, Tokyo, Japan) and fresh carrot. The collected eggs were placed on a piece of moist cotton in plastic dishes (50 mm diameter, 12 mm depth) and continuously maintained under LD conditions (Goto and Nagata 2022).

For experiments, first instar nymphs were kept in LD conditions or were transferred to short-day (SD) conditions (12-h light:12-h dark, 25.0 ± 1.0 °C). Adult females reared under LD conditions lay eggs destined to direct development (non-diapause), whereas those under SD conditions lay eggs destined to enter diapause (Goto et al. 2008). In the present study, females reared under LD and SD conditions were referred to as LD and SD females, respectively. Eggs laid by LD and SD females were referred to as LD and SD eggs, respectively.

Assessment of diapause status

Single adult females within one day after adult emergence were kept with two or three adult males in a plastic container (80 mm diameter, 140 mm height), and the eggs laid by each female were collected every two days until 10 days after adult emergence. Non-diapause eggs absorbed water 6–8 days after egg laying at 25 °C, and thereby the lengths of the major and minor axes of the egg became 1.1–1.3 times longer, whereas the diapause-destined eggs entered diapause before the absorption of water and thus did not change their sizes (Masaki 1960; Goto et al. 2008). In the present study, we maintained eggs under LD conditions and assessed their diapause status 13 days after egg laying under a stereomicroscope. Eggs that had absorbed water were considered as non-diapause, whereas those that had not were assumed to have entered diapause (Shiga and Numata 1997; Goto et al. 2008).

Hemolymph preparation for JH III and 20E quantification

We measured JH III and 20E concentrations in the hemolymph of adult females. A single decapitated female, 10 days after adult emergence, was placed head down in a perforated 0.5-mL tube inserted in a 1.5-mL silicon-coated microcentrifuge tube (Watson, Tokyo, Japan). The tube was centrifuged at 100×*g* for 5 min at 4 °C, and the collected hemolymph was stored at − 80 °C until use.

According to previous reports (Yamakawa et al. 1989; Zhou et al. 2011; Ando et al. 2020), hemolymph samples equivalent to 5–9 µL of the hemolymph of three or four individuals were mixed in a tube, and 500 µL of HPLC-grade methanol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was added. The sample was extracted with 250 µL of chilled HPLC-grade isooctane (FUJIFILM Wako Pure Chemical Corporation), and the isooctane phase was transferred to a new tube after centrifugation at 12,000×*g* for 5 min at 4 °C. This process was repeated three times. The pooled isooctane phase containing JH III was dried using a vacuum evaporator (CVE-2200, EYELA, Tokyo, Japan) at room temperature. The methanol phase containing 20E was dried in a vacuum evaporator at 40 °C. JH III and 20E samples were suspended in 30 µL of methanol and transferred to dedicated tubes (Autosampler vial #186000385C; Waters, Milford, MA, USA). The samples were stored at − 80 °C until use.

Egg preparation for JH III and 20E quantification

We measured the amounts of JH III and 20E in eggs. LD and SD eggs were collected immediately (0 day) and 1, 2, 3, 5, 7, 10, and 13 days after egg laying. SD eggs were also collected 20 and 30 days after egg laying. For JH III and 20E extraction, 50–70 and 10–50 eggs were used, respectively.

JH III was extracted according to the method described by Ando et al. (2020). Eggs were homogenized in 200 µL of methanol, and the homogenate was transferred to a 1.5-mL silicon-coated microcentrifuge tube. This process was repeated. After centrifugation at 1200×*g* for 15 min, the supernatant was transferred to a new 1.5-mL silicon-coated microcentrifuge tube. The samples were mixed with 100 µL of 2% NaCl solution and 200 µL of HPLC-grade hexane (FUJIFILM Wako Pure Chemical Corporation). After centrifugation at 12,000×*g* for 5 min at 4 °C, the hexane layer was transferred to a new 1.5-mL silicon-coated microcentrifuge tube. Hexane extraction was repeated three times. The samples were dried in a vacuum evaporator at room temperature, and 30 µL of methanol was added to each sample. The samples were stored at − 80 °C until use.

Extraction of 20E was performed according to the method described by Yamakawa et al. (1989). Eggs were homogenized in 200 µL of HPLC-grade acetonitrile (FUJIFILM Wako Pure Chemical Corporation), and the homogenate was transferred to a 1.5-mL silicon-coated microcentrifuge tube. This process was repeated. After centrifugation at 1,200×*g* for 15 min, the supernatant was transferred to a new 1.5-mL silicon-coated microcentrifuge tube. The samples were added to 200 µL of hexane, and the hexane layer was discarded to remove lipids. This process was repeated, and the samples were dried using a vacuum evaporator at 40 °C. The samples were suspended in 200 µL of methanol. The lipids in the samples were removed using 200 µL of hexane. The methanol layer was dried using a vacuum evaporator at 40 °C, and 20 µL of methanol was added to each sample.

Quantification of JH III and 20E

UPLC–MS/MS (ACQUITY UPLC H-Class, Xevo TQ-S micro, Waters) and a C18 column (ACQUITY UPLC BEH C18 Column, 2.1 × 100 mm, 1.7 µm particle size, Waters) were used to detect 20E and JH III with MassLynks software (Waters). The operating conditions for JH III detection were set according to the method described by Ando et al. (2020). The solvent for the C18 column was water:methanol (2:8) and the flow rate was 0.2 mL/min. The mass spectrometer settings were electrospray positive with a desolvation temperature of 350 °C. The operating conditions for the detection of 20E were set according to the method described by Batz et al. (2019). The mobile phase flow rate was 0.37 mL/min with a binary mobile phase of 0.1% formic acid in acetonitrile and 0.1% formic acid in water. The initial conditions were 1:99 acetonitrile:water ratio, followed by isocratic flow for 0.3 min. At 0.3 min, a linear gradient from 1:99 to 99:1 acetonitrile:water was applied over 4.2 min, followed by 1.0-min isocratic flow at 99:1 acetonitrile:water, after which the mobile phase returned to 1:99 acetonitrile:water. The injection volumes were 5.0 µL. MS/MS analysis revealed the fragment ions of JH III and 20E at $m/z = 267.3 > 43.0$ and $m/z = 481.37 > 371.11$, respectively. The detection limits of the JH III and 20E were approximately 0.3 pg and 5.0 pg, respectively (data not shown). JH III and 20E were quantified using the absolute calibration method.

Application of JH III and 20E to adult females

We assessed the effects of JH III and 20E on maternal decisions regarding embryonic diapause. Although no JH III and 20E application experiments have been conducted in *D. nigrofasciatus*, topical application of JH III and injection of 20E were effective in various physiological events in several cricket species (Stout et al. 1998; Ishimaru et al. 2019; Jin et al. 2021; Pang et al. 2023). In the present study, adult SD females within one day after adult emergence were kept individually with two or three males, and the eggs laid by each female were collected every two days. On day 10, the females were chilled on ice for 5 min for immobilization, and 1 µL of JH III (Sigma-Aldrich, St. Louis, MO, USA) in acetone (FUJIFILM Wako Pure Chemical Corporation) (10 µg/µL) was applied to the ventral side of the abdomen, or 1 µL of 20E (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) in 10% isopropanol (FUJIFILM Wako Pure Chemical Corporation) (10 µg/µL) was injected into the ventral side of the abdomen using a glass capillary. Some crickets were treated with both (JH III and 20E). The control group received 1 µL of acetone (Control 1), 10% isopropanol (Control 2), or both (Control 3). After treatment, the females were kept under SD conditions for 6 days. The diapause status of deposited eggs was assessed.

Statistical analysis

The hemolymph JH III and 20E concentrations were analyzed using the Student's *t*-test. The diapause incidence before and after JH III and 20E treatments was analyzed using a two-sample *Z*-test for proportions. The amount of 20E during early embryonic development was analyzed using a two-way ANOVA. As no significant difference was detected in the photoperiod, no further analysis was performed. All statistical analyses were performed in Python 3.11, using libraries such as pandas, numpy, scipy, and statmodels.

Results

Photoperiodic regulation of diapause and oviposition profiles

The developmental trajectories of *D. nigrofasciatus* offspring differed between photoperiods. Most eggs laid by LD females were non-diapause, whereas those deposited by SD females were diapause (Fig. 1a). The diapause incidences were 3.7% and 98.9% under LD and SD conditions, respectively (Fig. 1b). The number of eggs deposited was higher in LD females than in SD females (Fig. 1c).

The JH III and 20E hemolymph concentrations in the long-day and short-day females

We measured the hemolymph concentrations of JH III and 20E in females 10 days after adult emergence (Fig. 2). The hemolymph concentrations of JH III and 20E in LD females (mean ± standard deviation; 2.0 ± 0.3 and 51.9 ± 13.8 pg/µL, respectively) were significantly higher than those in SD females (1.2 ± 0.1 and 19.2 ± 8.0 pg/µL, respectively) (Student's *t*-test, $p < 0.05$).

Effect of JH III and 20E treatments on diapause status

The high hemolymph concentrations of JH III and 20E in LD females prompted us to assume that the phenotypes of LD females were caused by these hormones. To investigate this, we treated SD females with JH III, 20E, or both. Irrespective of the treatment, all females laid diapause eggs (Fig. 3a, b). Hormonal treatment did not increase the number of eggs deposited (Fig. 3c). We also treated females with low concentrations of JH III (1 µg) and 20E (1, 10, and 100 ng), but the diapause incidence was not affected (data not shown).

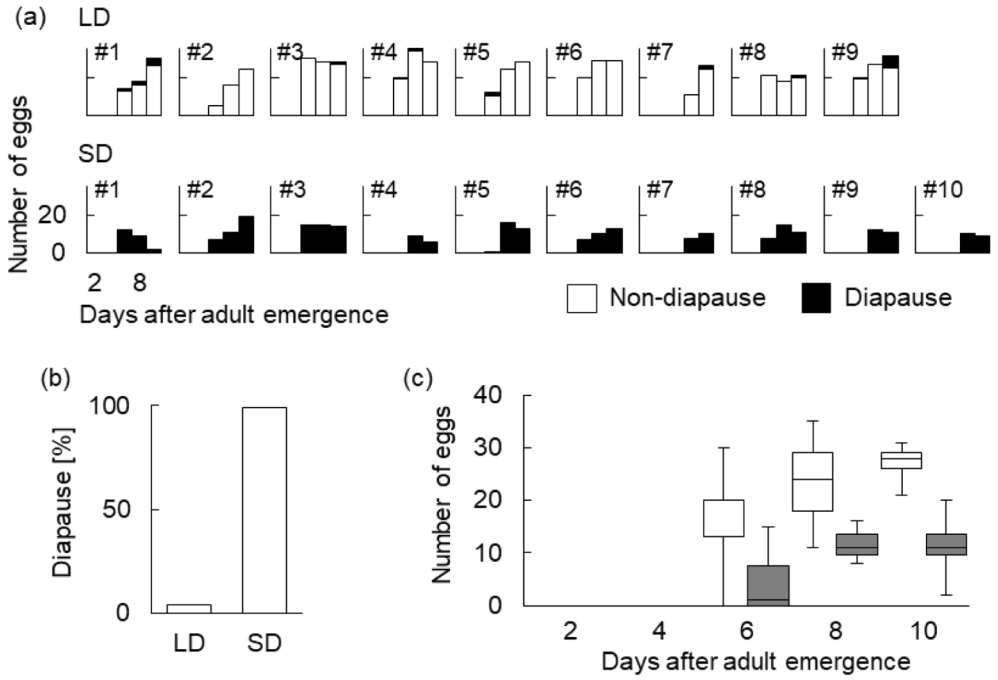

Fig. 1 Photoperiodic regulation of non-diapause and diapause egg production in *Dianemobius nigrofasciatus*. **a** Temporal changes in the number of non-diapause (open columns) and diapause (closed columns) eggs laid by each adult female under long-day (LD) and short-day (SD) conditions. **b** Diapause incidence under LD and SD conditions ($n = 593$ and 273, respectively). **c** Temporal changes in the number of eggs deposited by females under LD (open boxes) and SD (shaded boxes) conditions ($n = 9$ and 10, respectively). The line inside each box represents the median, and the top and bottom represent the 75 and 25 percentiles, respectively. The lines extending above and below the box indicate the maximum and minimum values, respectively. Data shown in **b** and **c** are from **a**

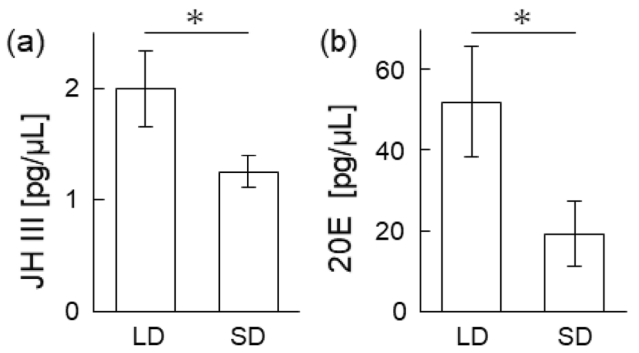

Fig. 2 Hemolymph concentrations of JH III (**a**) and 20E (**b**) in *Dianemobius nigrofasciatus* adult females under long-day (LD) and short-day (SD) conditions (mean ± standard deviation; $n = 3$). Hemolymph was collected 10 days after adult emergence. Student's *t*-test detected a significant difference ($^*p < 0.05$)

Amounts of JH III and 20E in eggs

We measured the JH III and 20E amounts in LD and SD eggs during early embryonic development. Figure 4a shows the temporal and photoperiodic changes in the amount of 20E in the eggs. A two-way ANOVA detected a significant difference in "day" ($p < 0.05$), but not in "photoperiod" and "interaction" ($p > 0.05$). Although we extracted JH III from the eggs 0, 1, 2, and 3 days after egg laying, their JH III amounts were lower than the detection limit.

To validate our methodology, we further investigated the temporal changes in JH III and 20E amounts during embryonic development (Fig. 4b). The LD eggs illustrated the dynamic nature of 20E amounts, and three distinct stages were detected: a small increment from days 0–3, a gradual decrease from days 5–10, and a drastic increase on day 13, several days prior to hatching. In contrast, the SD eggs gradually increased the 20E amount until day 5, and the amount was maintained thereafter. We detected JH III on day 10 (0.16 ± 0.05 pg/egg) and 13 (0.04 ± 0.02 pg/egg) in the LD eggs, but not in other days. JH III was undetectable in SD eggs.

Discussion

In this study, we investigated the possible involvement of JH III and 20E in maternal decisions regarding embryonic diapause in *D. nigrofasciatus*. Concentrations of JH III and 20E in the hemolymph of female adults were lower under short-day conditions than under long-day conditions. The reduction in JH and 20E concentrations is a typical response to short daylength in insects with reproductive diapause

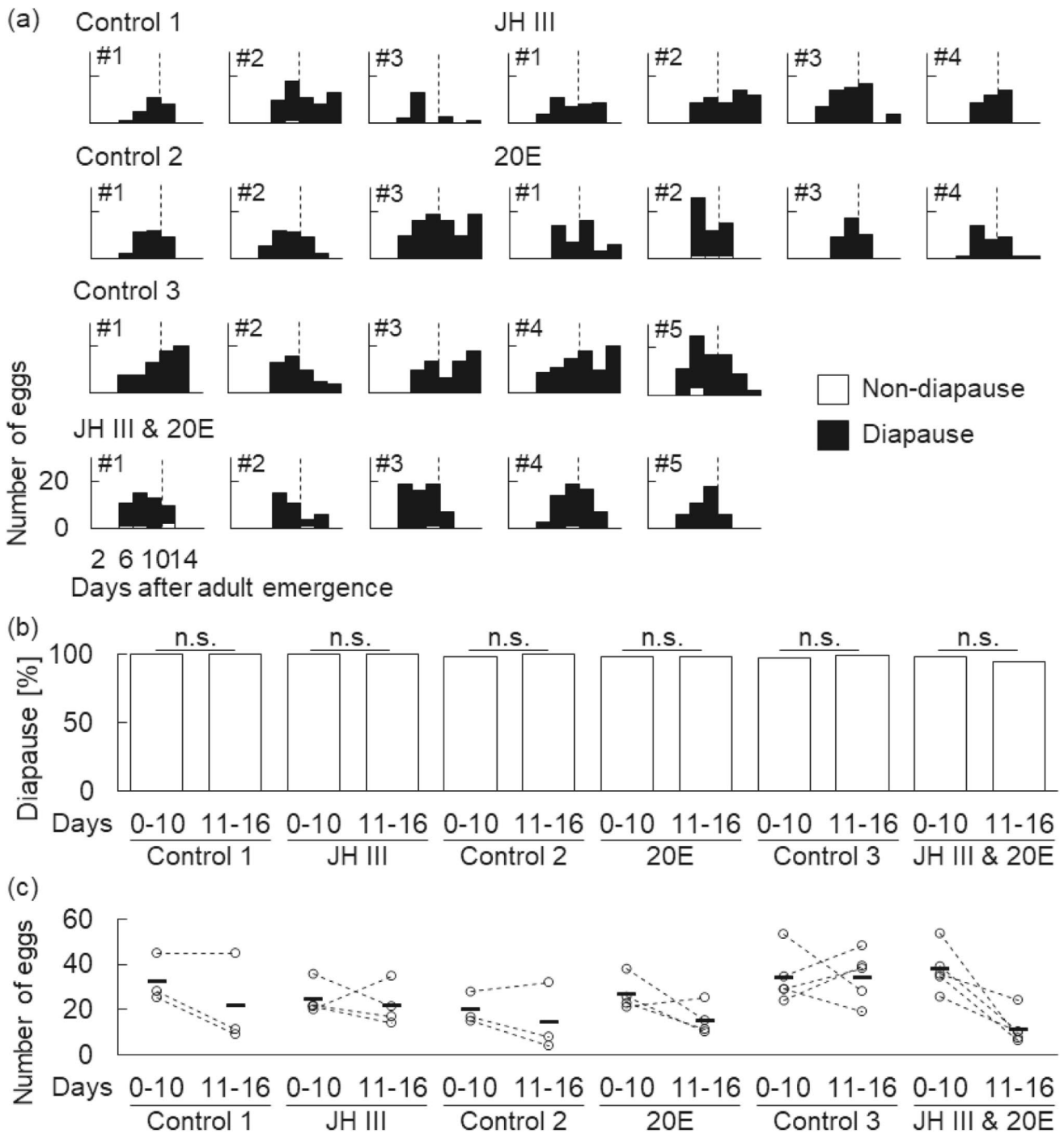

Fig. 3 Temporal changes in the number of eggs and their developmental status before and after hormonal treatment in the short-day females of *Dianemobius nigrofasciatus*. **a** Temporal changes in the number of non-diapause (open columns) and diapause (closed columns) eggs deposited. Hormonal treatments were performed 10 days after adult emergence (shown in the vertical broken line). Control 1: application of 1 µL of acetone, JH III: application of 1 µL of JH III in acetone (10 µg/µL), Control 2: injection of 1 µL of 10% isopropanol, 20E: injection of 1 µL of 20E in 10% isopropanol (10 µg/µL), Control 3: application of 1 µL of acetone and injection of 1 µL of 10% isopropanol, JH III & 20E: application of 1 µL of JH III in acetone (10 µg/µL) and injection of 1 µL of 20E in 10% isopropanol (10 µg/µL). **b** Diapause incidence before (0–10 days) and after (11–16 days) hormonal treatment in females under short-day conditions. The two-sample *Z*-test for proportions revealed no statistical significance (n.s., $p \geq 0.05$). **c** Number of eggs deposited by each female before and after hormonal treatment. The values for each female are connected by broken lines. Thick black bars indicate the mean of the total number of eggs. Data shown in **b** and **c** are from **a**

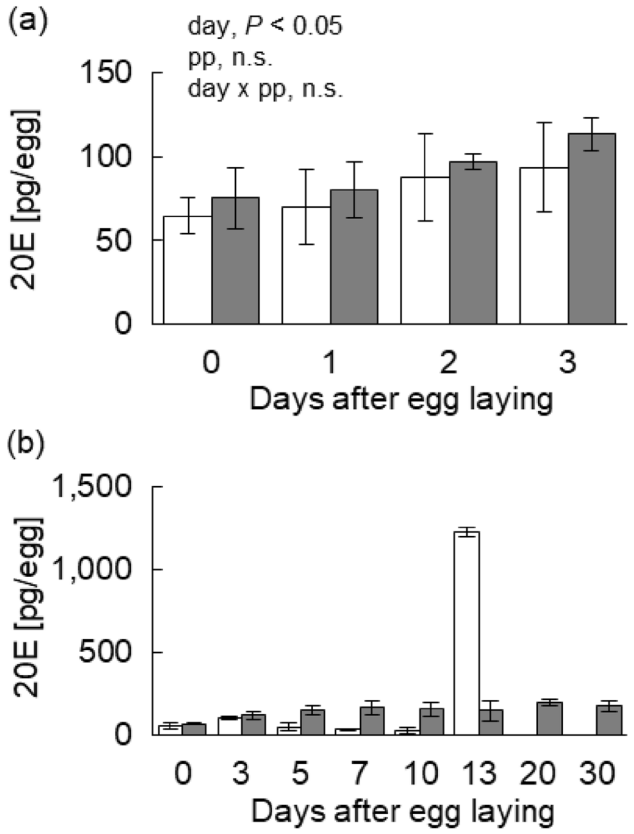

Fig. 4 Temporal changes in the amount of 20E in long-day (LD, non-diapause-destined) and short-day (SD, diapause-destined) eggs during embryonic development in *Dianemobius nigrofasciatus*. **a** Changes in the amount of 20E in LD (open columns) and SD (shaded columns) eggs during early embryonic development (mean ± standard deviation; $n = 4$ and 3 for LD and SD eggs, respectively). LD and SD eggs were collected 0, 1, 2, and 3 days after egg laying. Results of the two-way ANOVA of day, photoperiod (pp), and their interaction are shown. "n.s." indicates no significant difference ($p > 0.05$). **b** Temporal changes in the amount of 20E throughout embryonic development. Open and shaded columns indicate LD and SD eggs, respectively (mean ± standard deviation; $n = 3$ for each). LD and SD eggs were collected 0, 3, 5, 7, 10, and 13 days after egg laying. SD eggs were also collected 20 and 30 days after egg laying

(Richard et al. 1998; Denlinger et al. 2012); however, *D. nigrofasciatus* does not enter reproductive diapause. In *N. vitripennis*, which exhibits maternal regulation of larval diapause, a reduction in the hemolymph JH III is characteristic of diapause producers (Mukai et al. 2022). The application of small amounts of JH III (10–100 ng) immediately inhibited the transition from non-diapause to diapause production. Thus, JH III is a causal factor for maternal decisions regarding the developmental trajectory of offspring in this species. However, in *D. nigrofasciatus*, topical application of a much higher amount of JH III (10 µg) showed no effect on maternal decisions, although we adopted an identical methodology as in *N. vitripennis*. Injection of 20E also did not affect maternal decisions. The effect of 20E injection on maternal decisions has not been conducted in any other species, but the concentration we adopted (10 µg) was also higher than the conventional 20E application experiments in other crickets (0.5–3 µg) (Ishimaru et al. 2019; Jin et al. 2021).

In two locusts, *C. terminifera* and *L. migratoria*, the mother locusts were assumed to regulate embryonic diapause by controlling the amount of ecdysteroids transmitted into the eggs (Gregg et al. 1987; Tawfik et al. 2002). We investigated whether the concentrations of JH III and 20E in the hemolymph of female adults were reflected in the amounts of JH III and 20E transmitted to LD and SD eggs in *D. nigrofasciatus*. Our results indicate that there was no difference in the amount of 20E between the LD and SD eggs on day 0, and JH III was not detected in these eggs. Mother crickets do not appear to regulate embryonic diapause by controlling the amounts of JH III and 20E transmitted to the eggs. Thus, we found no evidence for the possible involvement of JH III and 20E in maternal decisions regarding embryonic diapause in the present study.

In *B. mori*, 20E sharply increases in non-diapause eggs but remains low in diapause eggs (Ohnishi et al. 1971; Sonobe and Yamada 2004). Treatment with 20E terminates embryonic diapause and resumes embryogenesis in *B. mori* and *L. migratoria* (Makka et al. 2002; Kidokoro et al. 2006). In contrast, a higher abundance of 20E appears to be important for initiating and maintaining diapause in the gypsy moth *Lymantria dispar* and ground cricket *Allonemobius socius* (Lee and Denlinger 1996, 1997; Lee et al. 2002; Reynolds and Hand 2009). JH is also involved in embryonic diapause. Pharate first-instar larval diapause of the mosquito *Aedes albopictus* appears to be induced by the low abundance of JH III (Poelchau et al. 2013; Batz et al. 2019). The JH analog pyriproxyfen terminates diapause in this species (Suman et al. 2015). Thus, JH III or 20E are important for regulating embryonic diapause in these species. However, in *D. nigrofasciatus*, JH III was not detected in the LD and SD eggs on days 1–3, and 20E amounts were not different between LD and SD eggs. These results suggest that JH III and 20E are not important for inducing embryonic diapause in this species. The hormonal mechanisms underlying embryonic diapause are distinct among insect species.

In summary, we found no evidence for the involvement of JH III and 20E in maternal decisions for embryonic diapause and diapause entry in *D. nigrofasciatus*. The factors regulating maternal induction of diapause remain unclear. To identify these factors, RNA-seq analyses of the mother's brain, ovaries, and eggs will provide important information. Changes in the brain would reveal maternal endocrinological factors, those in the ovary would clarify how the mother transmits seasonal information to eggs, and those in the eggs would reveal direct factors regulating diapause entry. Additional omics approaches, such as proteomic, metabolomic, and small RNA-seq analyses, could elucidate

diapause-inducing factors. It may also be important to focus on diapause hormone, which is involved in maternal decisions in some species (Yamashita 1996; Sato et al. 1998; Uehara et al. 2011; Hao et al. 2019). Epigenetic processes, such as DNA methylation and histone modifications, may also be one of the key processes (Reynolds 2017).

Author contributions The authors contributed to the study conception and design. Material preparation, data collection, analysis, and the first draft of the manuscript were performed by YS. The authors commented on previous versions of the manuscript and approved the final manuscript.

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Data availability Data will be made available on request.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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