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Juvenile Hormone Biosynthesis in Diapause and Nondiapause Females of the Adult Blow Fly *Protophormia terraenovae*

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ABSTRACT—*In vitro* synthetic activities of juvenile hormones (JH) were examined using a radiochemical assay in diapause females and reproductive females of the blow fly, *Protophormia terraenovae*. Thin layer chromatography showed that products of the corpus allatum (CA) comigrated with a synthetic sample of JH III bisepoxide but neither with JH III nor methylfarnesoate. JH synthetic activities increased in females reared under LD 18:6 at 25°C, as the ovaries developed. The synthetic activities remained low in previtellogenic females reared under LD 12:12 at 20°C. Removal of the pars intercerebralis compelely prevented ovaries from development under reproductive conditions, and removal of the pars lateralis caused partial or full development of ovaries under diapause-inducing conditions. In these operated animals, the JH synthetic activities were not significantly different from those of the intact and sham-operated animals. The results indicate that the CA in *P. terraenovae* produces mainly JH III bisepoxide and a decrease in the JH production rate is a cause of diapause induction. PI neurons and PL neurons in the brain do not directly mediate changes in the JH production rate, but regulate ovarian development cooperatively with some unknown allatostatic and allatotropic factors.

Key words: juvenile hormone, JH III bisepoxide, reproductive diapause, blow fly, *Protophormia terraenovae*

INTRODUCTION

Endocrinological mechanisms controlling reproductive diapause have been examined in several orders of insects. One of the most thoroughly examined species is the Colorado potato beetle *Leptinotarsa decemlineata*, in which long-days cause reproduction while short-days induce diapause (de Wilde *et al*., 1959). When the corpus allatum (CA) was removed from long-day beetles, reproductive activities ceased and beetles entered diapause. This suggests that lack of juvenile hormone (JH) is responsible for diapause (de Wilde and de Boer, 1961). This suggestion was verified by direct measurements of the activity of the CA by radiochemical assays *in vitro* (Kramer, 1978; Khan *et al*., 1982). The JH biosynthetic rate was high in long-day reproducing adults, but it was low during diapause under short-day conditions.

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For induction of diapause, insects receive environmental signals such as photoperiod and temperature, which are processed by the nervous system so that hormonal production is inhibited to cause developmental arrests. The neural elements processing information from reception of photoperiod and temperature to the control of hormonal events have not been well understood. Inhibitory roles of the nervous connection from the brain to the CA in oocyte development have been shown in many species by transection of the nervi corporsis allati (NCA) or by extirpation of the neurosecretory cells in the brain (Denlinger, 1985). After these operations the ovaries developed under diapause-inducing conditions. It is most probable to interpret the results that these operations liberate CA suppression and an increase in JH production causes oocyte development. However, it is also possible that the operations prevent other pathways necessary for diapause induction. To understand neural and endocrine elements to inhibit oocyte development under diapause-inducing conditions, measurements of JH synthetic activities after these operations are necessary. In *L. decemlineata* the measurement of activities of innervated and den-

ervated CA under various experimental conditions revealed that the CA is controlled by signals arriving along the neural as well as the humoral pathway (Khan *et al*., 1983). But humoral influence was subordinate to an inhibitory effect sustained by intact nerve connections with the brain. Further selective lesions of the brain combined with radiochemical assays of the CA demonstrated that regions involved in inhibition of JH production for ovarian development is in the pars lateralis (Khan *et al*., 1986). Although reproductive diapause is found in a variety of insects, the underlying mechanisms have been extensively examined only in *L. decemlineata*. To understand how environmental stimuli are processed to control reproductive diapause, careful studies in more species are necessary.

The blow fly *Protophormia terraenovae* living in the subfrigid zone, enters adult diapause with an arrest of vitellogenesis in females and with halts of mating behavior in males under short-day conditions at a low temperature (Numata and Shiga, 1995; Tanigawa *et al*., 1999). Transplantation of the CA or application of JH analogue (JHA) causes reproduction under short-day conditions (Matsuo *et al*., 1997). Further, two kinds of cerebral neurons controlling ovarian development have been identified. These are pars intercerebralis (PI) neurons, which promote ovarian development in diapause-averting conditions and pars lateralis (PL) neurons, which suppress ovarian development in diapause-inducing conditions (Toyoda *et al*., 1999; Shiga and Numata, 2000).

In the present study, we examined *in vitro* synthetic activities of JH by the CA. The results were compared between diapause females and reproductive females. Then, the JH synthetic activity was measured after removal of the PI or PL to know whether PI and PL neurons control reproductive diapause by changing JH production rates or mediating some other pathway.

MATERIALS AND METHODS

Insects

Adults of *P. terraenovae* (Diptera: Calliphoridae) were collected at a swine farm in Obihiro City (42° 56'N, 143° 12'E), Japan. Their progeny was maintained in laboratory cultures under a photoperiod of LD 18:6 (18 h light and 6 h darkness) at 25±1°C as previously described (Numata and Shiga, 1995). For the present study larvae, pupae and adults of each group were reared in constant conditions either of LD 18:6 and 25±1°C for reproduction, or of LD 12:12 and 20±1°C for diapause induction. Larvae were fed on beef liver or an artificial diet (Tachibana and Numata, 2001). Only females were used for the experiments. Females were collected on the day of adult emergence (day 0), and 7–15 individuals were reared without males in a plastic pot (15 cm diameter, 9 cm depth) covered with nylon net. They were fed on sucrose, a piece of beef liver and water.

JH biosynthesis by the CA *in vitro*

The radiochemical assay for measuring JH biosynthesis by the CA was employed basically according to Okuda *et al*. (1996). Flies were anesthetized on ice for 5 min before dissection. Tissue preparation was made in three kinds: 1) the CA alone, 2) the CA connected to the hypocerebral ganglion including the corpus cardiacum (CAHG), and 3) CAHG connected to fused cephalic ganglia including the brain (without lamina of the optic lobe) and subesophageal ganglion (CAHGBr). Dissection was made in the minimum essential medium with Hanks' salts with L-glutamine without sodium bicarbonate (MEM, Gibco BRL, NY) containing 20 mM HEPES and 5 ppm Tween 8 (pH=7.2) on ice. The aorta, tracheoles, and fat bodies were carefully removed from the tissue. Single CA, CAHG or CAHGBr was incubated in a siliconized glass tube containing 30 µl of the MEM supplemented with 4% L-[methyl-^{3}H]-methionine (specific activity of 2.59–3.145 TBq/mmol, Amersham, Buckinghamshire; NEN, MA) for 2 h at 30°C. After the incubation, 100 µl of hexane was added to extract the synthesized products. An aliquot of 50 µl upper hexane phase was collected and applied to a silica thin layer chromatography (TLC) plate (Merck, Darmstadt). Unlabeled JH III (Sigma, MO), JH III bisepoxide (JHB_3) and methyl farnesoate (MF) were also mounted on different lanes of the TLC plate as markers. JHB_3 was synthesized according to Richard *et al*. (1989). MF was synthesized according to Yin *et al*. (1995). The TLC plate was developed with a mixture of hexane and ethyl acetate, 1:1. After development spots of markers were visualized under UV light (280 nm). Each lane was sectioned every 5 mm and the radioactivity was determined in a scintillation liquid, ACS II (Amersham, VA) with a liquid scintillation counter (Beckman, CA).

Surgery of the brain

Operations were made one day after adult emergence (day 1). The methods used for brain surgery are described in Toyoda *et al*. (1999) and in Shiga and Numata (2000). After the operations, 10–15 females were isolated in a plastic cage with sucrose, beef liver

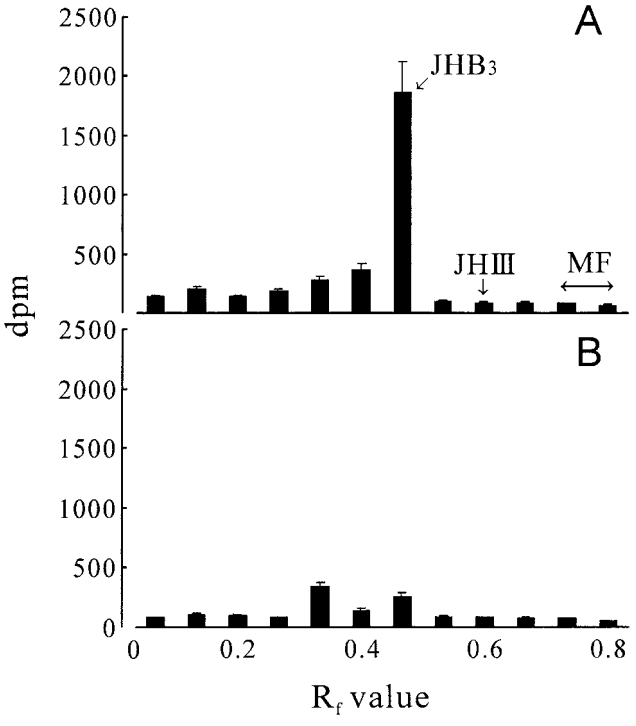

Fig. 1. Thin-layer chromatography of hexane extracts of synthetic products by the CA in adult females *Protophormia terraenovae* on day 6 under LD 18:6 at 25°C (**A**) and on day 11 under LD 12:12 at 20°C (**B**). Columns and vertical bars are mean values of 4–5 determinations with standard errors of the mean. Abscissa denotes R_f values. Arrows indicate R_f of nonradioactive JH III bisepoxide (JHB_3), JH III and methyl farnesoate (MF). Note that high radioactivity occurs in the same R_f value as JHB_3 under LD 18:6 at 25°C.

and water. On day 11 under LD 12:12 at 20°C or on day 6 under LD 18:6 at 25°C, flies were first subjected to dissection of the CA or CAHG for the radiochemical assay. After dissection, neurons in the PI and PL were back-filled from the cardiac recurrent nerve for autopsy (Shiga and Numata, 2000) and ovarian stages were examined. Five mg/ml of 3000 kDa dextran tetramethylrhodamine and biotin (D-7162, Molecular Probes, OR) was used for back-fills through the cardiac recurrent nerve (Shiga *et al*., 2000). Observation was made under a fluorescent microscope with a filter set (U-MWIG, Olympus, Tokyo). A CCD camera (CoolSNAP, Olympus, Tokyo) digitalized microscopic images. Digital images were processed by Corel Draw 9.0 (Corel Corp., Ottawa) and Adobe Photoshop 5.0 (Adobe System Incorporated, Tokyo).

Ovarian stages

Females were classified into three groups according to ovarian developmental stages: previtellogenic ovaries, vitellogenic but not fully developed ovaries, and fully developed ovaries. Females with previtellogenic ovaries were considered to be in diapause, and those with vitellogenic ovaries, both not fully developed ovaries and fully developed ovaries, were to be in reproduction.

RESULTS

Biosynthetic products by the CA *in vitro*

Fig. 1 shows TLC separation of the products of the CA. Under reproductive conditions of LD 18:6 at 25°C, high radioactivity occurred at an R_f value of 0.47 on day 6 when most females had vitellogenic ovaries. A synthetic sample of JHB_3 also occurred at the same R_f value. Radioactivities were low in other R_f values including those of JHIII or MF. Under diapause-inducing conditions of LD 12:12 at 20°C, radioactivities were low at all R_f values. No females had

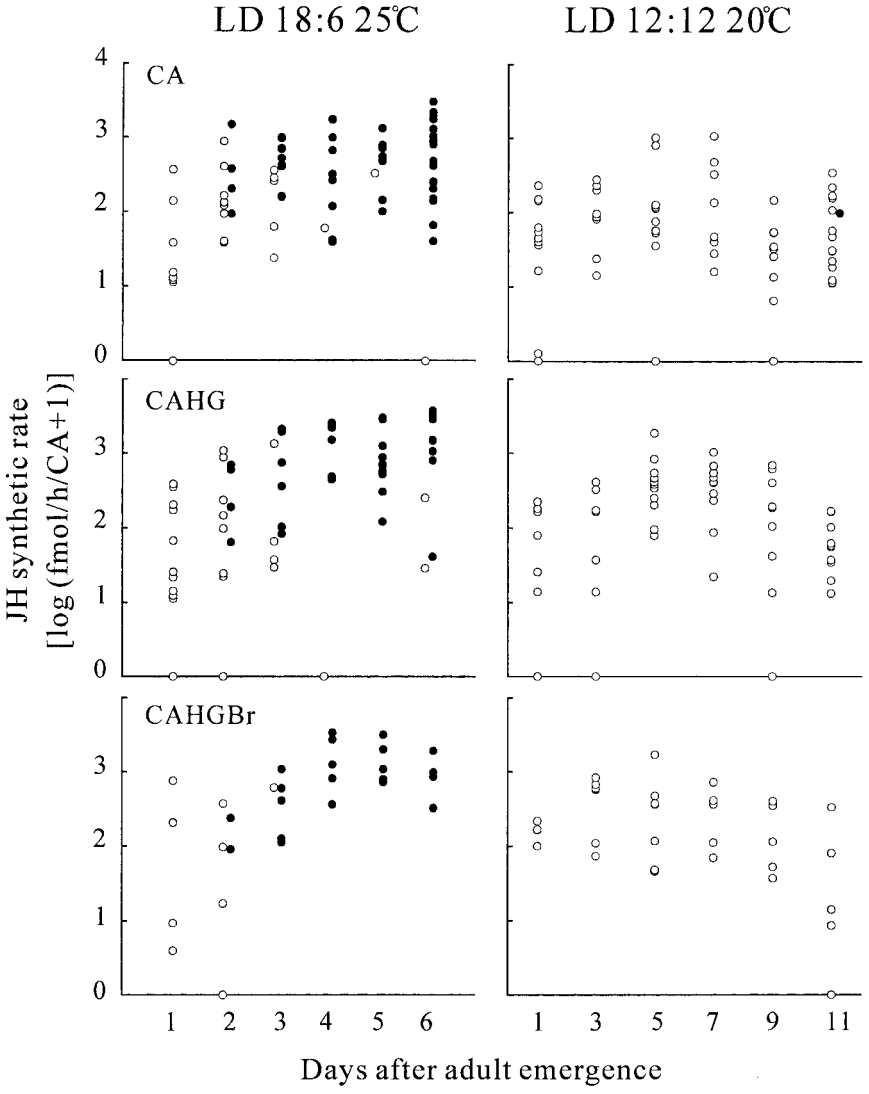

Fig. 2. Individual plots of JH synthetic rates and ovarian stages in intact adult females *Protophormia terraenovae* under LD 18:6 at 25°C and LD 12:12 at 20°C. The synthetic rates were calculated from the radioactivity at the same R_f value as JHB_3 and the values of the synthetic rate plus 1.0 are shown in the logarithmic scale of the ordinate. Open circles show females with previtellogenic ovaries and closed ones females with vitellogenic ovaries. The upper graphs show the products in the CA culture, the middle ones the products in the CAHG culture, and the bottom ones the products in the CAHGBr culture. Under LD 12:12 at 20°C all females had previtellogenic ovaries except for one on day 11 in the CA culture. n=4-22.

vitellogenic ovaries. The results suggest that the CA in *P. terraenovae* mainly synthesizes JHB_3 in the reproductive state. In the following experiments radioactivities at the same R_f value as JHB_3 were regarded as synthetic activity of JHB_3.

Comparison of JH synthetic rates between reproductive and diapause females

Synthetic activities of JHB_3 were examined in developmental courses under LD 18:6 at 25°C and LD 12:12 at 20°C (Fig. 2). Under LD 18:6 at 25°C vitellogenic ovaries were observed on day 2 and later. When the synthetic rate was measured in the CA incubation, it gradually increased as the ovaries developed. The synthetic rate was 12.1 fmol/h/CA (median, n=9, range=0-381.48 fmol/h/CA) on day 1. On day 6, 20 out of 22 females had vitellogenic ovaries and their synthetic rate was 832.3 fmol/h/CA (median, n=20, range=0-3154.3 fmol/h/CA). Two females had previtellogenic ovaries and their synthetic rate was at the noise level. The JHB_3 production rate on day 6 (n=22) was significantly larger than that on day 1 (n=9) (P<0.01, Mann-Whitney test). In contrast, under LD 12:12 at 20°C the production rate of JHB_3 was low throughout the experimental period and all females except for one on day 11 had previtellogenic ovaries. There were no significant differences in the production rate between day 1 (median=41.5 fmol/h/CA, n=12, range=0-230.6 fmol/h/CA) and day 11 (median=46.8 fmol/h/CA, n=15, range=10.3-344.6 fmol/h/CA) (P>0.05, Mann-Whitney test). When the JHB_3 synthetic rates were measured by the CAHG or CAHGBr culture, profiles of the synthetic rate were similar to the CA culture. Both under LD 18:6 at 25°C and LD 12:12 at 20°C, there were no significant differences in the synthetic rate among the CA, CAHG and CAHGBr culture on each day (P>0.05, Kruskal-Wallis test).

Effects of the PI or PL removal on JH production rates

The regions of the PI or PL were bilaterally removed, and JHB_3 production rates and ovarian stages were examined. Because no differences in the production rates were detected among the CA, CAHG and CAHGBr culture, the production rate was examined mainly by the CA culture. Backfills were also made after the experiment to check success of the operation. In intact individuals a large number of somata were stained in the PI (Fig. 3A). In the PL we counted two types of neurons: 2 PL-c neurons with contralateral axons to the retrocerebral complex (RC) and 4–7 large type PL-i neurons with ipsilateral axons to the RC per hemisphere. There were many small type PL-i neurons (Shiga *et al*., 2000), which were not counted in the current study. After removal of the PI, individuals with roughly two thirds or more of PI somata disappeared and with at least three (a total number in both hemispheres) of large type PL-i neurons left were used for the analysis (Fig. 3B). Of 22 individuals that fulfilled the above criteria 17 individuals completely lost PL-c neurons after removal of the PI, because axonal pathways of PL-c neurons to the contralateral side were interrupted. In the PL removal individuals with neither PL-i nor PL-c somata stained and with most PI somata left were used for the analysis (Fig. 3C).

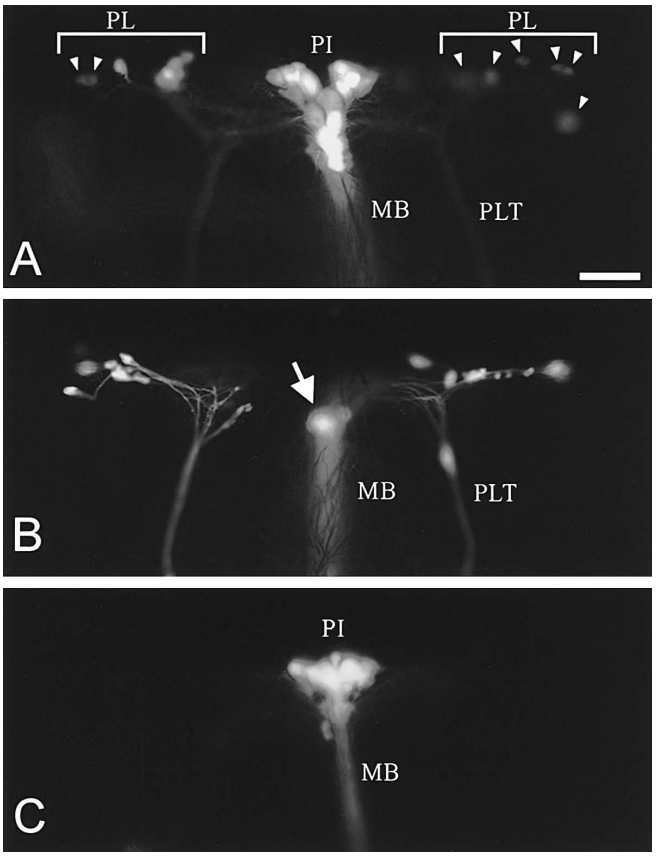

Fig. 3. Photomicrographs of brain neurons back-filled from the cardiac-recurrent nerve of adult females, *Protophormia terraenovae* (frontal view of the dorsal part of the whole-mount brain). **A** An intact brain. A cluster of somata in the pars intercerebralis (*PI*) and somata in the pars lateralis (*PL*) were stained. Arrowheads indicate somata out of focus. **B** A brain with the PI removed. A few PI somata were left (arrow) and PL somata were labeled as in intact brains. **C** The brain with the PL removed. Only somata in the PI and axons in the median bundle (*MB*) were labeled. *PLT*, posterior lateral tract. *Scale*, 100 μm.

Fig. 4 shows effects of PI or PL removal on ovarian stages and on JHB_3 synthetic rates. Under LD 18:6 at 25°C, 20 out of 26 individuals in the intact group, all (n=8) in the sham-operated group and all (n=10) in the PL removal group had vitellogenic ovaries. In vitellogenic females, percentage of females with fully developed ovaries in the PL removal group (60%) was lower than in the intact (95%) and sham-operated groups (87.5%). In the rest of vitellogenic females, yolk deposition was partly observed in ovarioles. In the PI removal group all individuals (n=16) had previtellogenic ovaries. PI removal completely interrupted ovarian development. JHB_3 production rates, however, showed no significant differences among the intact, sham-operated, PI removal and PL removal groups in LD 18:6 at 25°C (P>0.05, Kruskal-Wallis test). Under LD 12:12 at 20°C, 9 out of 14

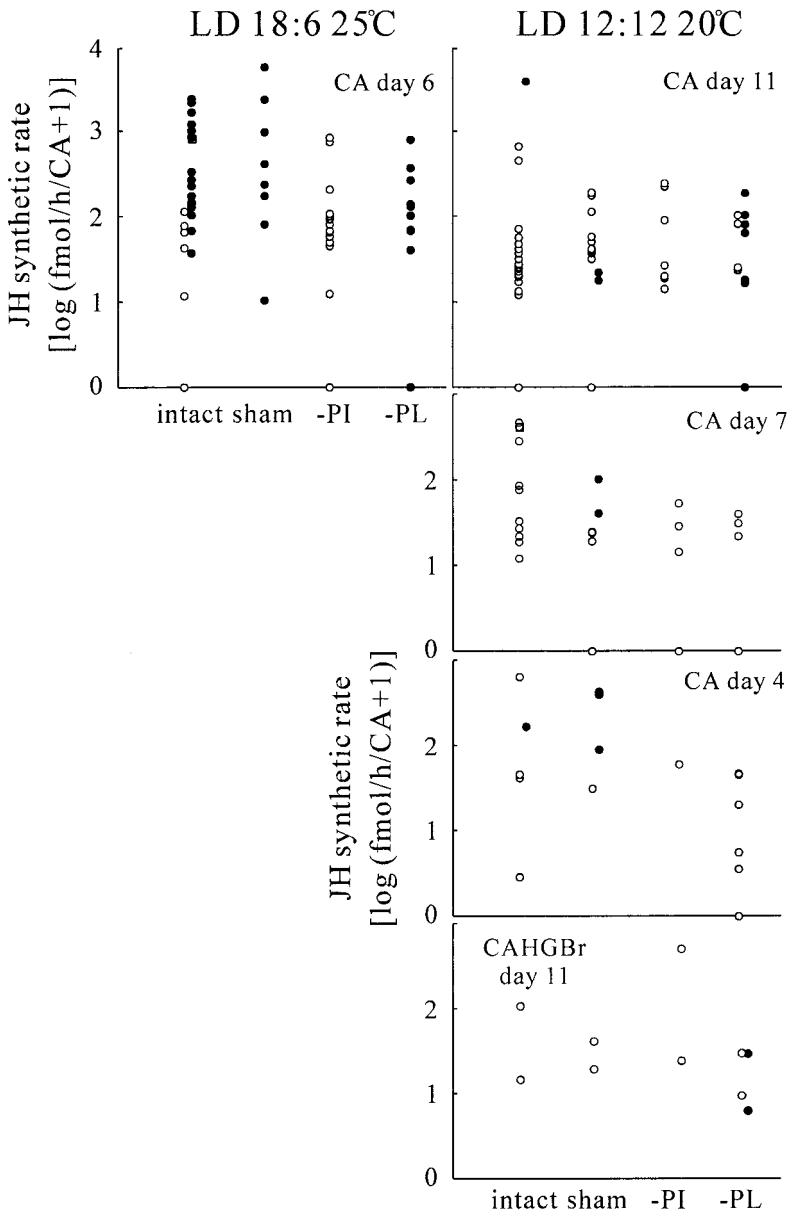

Fig. 4. Effects of removal of the pars intercerebralis (*PI*) or pars lateralis (*PL*) on JHB_3 synthetic rates in adult females *Protophormia terraenovae* under LD 18:6 at 25°C and LD 12:12 at 20°C. Abscissa shows experimental groups of the intact, sham-operation (*sham*), removal of the PI (*-PI*) and removal of the PL (*-PL*). Days for the measurement and cultured organs are indicated in each graph. See also Fig. 2 for further explanations.

females developed the ovaries after removal of the PL, although most females in the intact, sham-operated and the PI removal groups had previtellogenic ovaries. In vitellogenic females in the PL removal group, 44.4% (n=9) had fully deloped ovaries. This percentage was low compared with those in the intact or sham-operated groups under LD 18:6 at 25°C. PL removal averted diapause induction but was not able to cause full development of ovaries in some females as shown by Shiga and Numata (2000). In LD 12:12 at 20°C also, JHB_3 production rates were not different among the intact, sham-operated, PI removal and PL removal groups (P>0.05, Kruskal-Wallis test). In the PL removal group under LD 12:12 at 20°C the production rates were not different between females with previtellogenic ovaries and those with vitellogenic ovaries (P>0.05, Mann-Whitney U test). In vitellogenic females of the PL removal group JHB_3 production rates were not different between females with fully developed ovaries and those with partly developed ovaries both under LD 12:12 at 20°C and under LD 18:6 at 25°C (P>0.05, Mann-Whitney U test).

JHB_3 production rates were also examined in earlier stages on day 7 and day 4, or by the CAHGBr culture on day 11 (Fig. 4). However, the production rate of JHB_3 was not different among the intact, sham-operated, PI removal and PL removal groups (P>0.05, Kruskal-Wallis test). Production rates neither of JH III nor MF were consistently low

in any experiments (data not shown).

DISCUSSION

Under reproductive conditions, TLC analysis showed high radioactivity only at the same R_f value as methyl 6, 7; 10, 11-bisepoxy-3, 7, 11-trimethyl-(2E)-dodecenoate, JHB_3. No radioactivities occurred at the R_f value of JH III or MF. Although detail physicochemical analyses are needed for identification of the chemical structure of JH in *P. terraenovae*, this species seems to biosynthesize exclusively JHB_3. A combination of the radiochemical assay and various chromatographic analyses, such as TLC, high performance liquid chromatography and gas chromatography-mass spectrometry has demonstrated that the CA and ring glands in cyclorrhaphous flies predominantly biosynthesize JHB_3 (Yin, 1994). JHB_3 has been identified first in *Drosophila melanogaster* (Richard *et al*., 1989) and later in other cyclorrhaphous species (Yin and Stoffolano, 1997). In addition to JHB_3, JH III and MF are also found as products in the CA in *Phormia regina* (Yin *et al*., 1995) and *Neobellieria bullata* (Bylemans *et al*., 1998). But in *Lucilia cuprina* (Lefevere *et al*., 1993), *Drosophila melanogaster* (Richard *et al*., 1989) and *Calliphora vomitoria* (Cusson *et al*., 1991) the production of JH III or MF is negligible. This is also true in *P. terraenovae*.

Matsuo *et al*. (1997) showed that removal of the CA arrested ovarian development under reproductive conditions, and applications of JHA or implantation of the active CA to diapause females caused reproduction in *P. terraenovae*. These results highly suggested that suppression of JH production and release in the CA is a direct cause for reproductive diapause in this species. The present study validates this supposition. JH production is high in reproductive females and low in diapause females in *P. terraenovae*. In regard with the reproductive diapause, JH synthetic activities of the CA or JH titers in the hemolymph have been examined in *Leptinotarsa decemlineata* (Khan *et al*., 1982), *Locusta migratoria* (Poras *et al*., 1983; Okuda *et al*., 1996), *D. melanogaster* (Saunders *et al*., 1990), *Bombus terrestris* (Larrere *et al*., 1993), *Pyrrhocoris apterus* (Hodková *et al*., 1996), *Plautia crossota stali* (Kotaki, 1999), *Culex pipiens* (Readio *et al*., 1999) and *P. terraenovae* in the present study. In all the species JH synthetic rates or titers are low in diapause adults compared with those in reproductive ones. Given that the rate of JH production by the CA is generally considered a factor with the greatest influence on hemolymph JH titers in adult insects (Tobe and Stay, 1985), the idea that adult diapause is caused by absences or low titers of JH could be accepted in a wide variety of insects.

In *P. terraenovae*, environmental signals of the short-day photoperiod and low temperature are processed in the nervous system to inhibit JH production in the CA to suppress ovarian development. Contrary, the long-day photoperiod and high temperature potentiate JH production to cause vitellogenesis. In our previous studies, surgical ablation revealed inhibitory roles of the PL neurons and stimulating roles of the PI neurons in ovarian development (Toyoda *et al*., 1999; Shiga and Numata, 2000). Fiber projections of these neurons postulate that the PL neurons innervating the CA might inhibit ovarian development by suppressing JH production, and the PI neurons terminating at the HG secrete humoral factors leading to JH production or some other pathway to cause ovarian development (Shiga *et al*., 2000). To examine the hypothesis, JH synthetic rates were measured after removal of the PL or PI neurons in the currenst study.

After removal of the PL, vitellogenesis occurred in many females under diapause-inducing conditions. Full development of ovaries, however, was not observed in all females as shown by Shiga and Numata (2000), and JH production rates were low through the adult stage. This suggests that PL neurons inhibit ovarian development via other mechanisms than suppression of JH synthesis. There must be unknown allatostatic mechanisms to keep low rates of JH production during diapause. PL neurons and unknown allatostatic factors cooperatively suppress ovarian development under diapause-inducing conditions in *P. terraenovae*. PL neurons might be involved in waning of JH effects on target organs by lowering of JH titers in the hemolymph. After removal of PL neurons there were low but some production rates of JH under diapause-inducing conditions. Liberation from PL neurons may potentiate effects of a small amount of JH in the hemolymph on target organs to cause vitellogenesis. Their ovaries, however, were not fully matured in some females, because a low level of JH might not be sufficient for full development of ovaries. Under diapause-inducing conditions transection of the nervus corporis allati, in which PL neurons send axons, caused vitellogenesis but about a half of vitellogenic females had partly matured ovaries (Matsuo *et al*., 1997). The results are similar to those after PL removal (Shiga and Numata, 2000; the present study).

Under diapause-inducing conditions application of JHA caused full development of ovaries (Matsuo *et al*., 1997), suggesting that an excess of JH may make up for inhibitory roles of the PL neurons. Although allatostatic functions have been shown in PL neurons in *Leptinotarsa decemlineata*, *Locusta migratoria* and *Diploptera punctata* (Khan *et al*., 1986; Rüegg *et al*., 1983; Couillaud *et al*., 1985), the PL neurons in *P. terraenovae* seem to have other roles in suppression of vitellogenesis. Allatostatic factors have not been identified in dipteran insects.

In *Bombus terrestris* adult diapause is induced by low rates of JH production. In this species diapause termination occurs earlier than increases in JH production rates. Diapause termination by carbon dioxide narcosis could also occur without increases in JH production rates (Larrere *et al*., 1993). In *P. crossota stali* transection of the nerve between the brain and complex of the CC and CA caused ovarian development first, and afterwards an increase in JH biosynthesis was observed (Kotaki, 1999). These studies

clearly indicate that a high rate of JH production is not necessarily required for diapause termination or aversion, and other factors also contribute to ovarian development in addition to an elevation of JH synthesis.

In *L. migratoria*, nerve-mediated inhibitions of JH production were observed in normal developmental cycles of ovaries (Horseman *et al*., 1994). When the nerve was transected during low rates of JH production, a progressive increase in JH biosynthesis occurred immediately. In the present study JH production rates were compared between the CA alone and that attached with the HG and brain. No significant differences in JH production rates were found among different organ complexes for incubation. JH production rates of the CA itself, separated from the HG and brain, were low under diapause-inducing conditions and high under reproductive conditions. It seems that *P. terraenovae* has different mechanisms for CA suppression.

Removal of the PI compleltely prevented ovarian development but JH production rates were high as in the intact and sham-operated groups. Ovarian development was suppressed even with active CA, suggesting that the PI neurons released substances that were necessary for ovarian development other than JH. There are factors stimulating the ovaries to secrete ecdysteroids, which are necessary for vitellogenin synthesis in *P. terraenovae* (Huybrechts and De Loof, 1982). The primary structure of an ovary ecdysteroidogenic hormone, which is a peptide that stimulates the ovaries to secrete ecdysteroids, has been identified in two or three pairs of PI neurons in *Aedes aegypti* (Brown *et al*., 1998). The PI neurons in *P. terraenovae* might also release ecdysteroidogenic hormones.

Based on our previous and current studies, suppression of JH synthesis is a cause for induction of reproductive diapause in *P. terraenovae*. PI neurons and PL neurons in the brain also regulate ovarian development by other pathways than control of JH production in the CA. PI, PL neurons, and unknown allatostatic or allatotropic factors cooperatively control adult diapause and reproduction. The mode of action of the PI and PL neurons in reproduction and diapause induction remains to be elucidated.

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