Analysis of neuroendocrine mechanisms underlying photoperiodic responses in the brown-winged green bug *Plautia stali*

(チャバネアオカメムシにおける光周性の神経内分泌機構の解析)

理学研究科

生物地球系専攻

平成 27 年度

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Contents

General Introduction

The daily rotation of the earth on its axis and annual rotation of the earth around the sun establish two fundamental environmental cycles, imposing the physiological rhythms on organisms. The daily rotation is responsible for the biological rhythms with a period of approximately 24 h named "circadian". By contrast, the annual rotation generates physiological reactions by organisms to day or night length that are named "photoperiodism". To adapt seasonal environmental changes, many organisms have evolved photoperiodism. Photoperiodism permits organisms to adjust their activity, development and reproduction for undergoing seasonally changing environments (Nelson et al., 2010).

The pioneering research of photoperiodism reported in plants and then the classic demonstrations of photoperiodism were reported in the strawberry root aphid *Aphis forbesi* (Marcovitch, 1923) and the silk moth *Bombyx mori* (Kogure, 1933). Since these early researches, the control of seasonal polyphenism such as diapause and seasonal migration has been extensively studied in insects (Sanders, 2002). Physiological mechanisms underlying photoperiodic responses comprise a "photoreceptor" distinguishing light from dark, a "photoperiodic clock" including measurement of the day or night length and counting of day numbers, and an "endocrine effector" releasing hormones regulating seasonal phenotypes (Saunders, 2002). The photoreceptor organs, endocrine organs and hormonal molecules have been shown in many species (Numata et al. 1997; Denlinger et al., 2005). Recently,

involvement of circadian clock genes shown in photoperiodic responses has been in dipteran, orthopteran and heteropteran insects (Pavelka et al., 2003; Sakamoto et al., 2009; Ikeno et al., 2010).

Although necessary organs and molecules for photoperiodic responses have been gradually revealed, it remains largely unsolved how the brain integrates photoperiodic information to turn on or off endocrine factors controlling seasonal phenotypes. In the previous studies, involvement of the brain or clock genes in control of photoperiodic phenotypes, such as reproductive organs, has been studied. However, it is unknown what the brain directly controls to cause seasonal phenotypes. To understand neuroendocrine mechanisms of photoperiodism, it is important to clarify how the brain controls endocrine effectors.

One of seasonal phenotypes controlled by photoperiod is diapause. Generally, during diapause, development is slowed or stopped in immature insects or reproduction is repressed in adult insects. Most insects sense changing environments to prepare for diapause well before unfavorable conditions (such as winter) arrive. During diapause, metabolism is typically depressed, so insects use less energy, and nutrient reserves are conserved in the body (Hahn and Denlinger, 2007). Diapause is obligatory for some species, typically those with a univoltine life cycle, so that every generation enters diapause at fixed stages in their life cycle. In most species with multivoltine life cycles, diapause is facultative and generations continue without diapause during favorable seasons, but during unfavorable seasons insects enter diapause.

In many species which enter diapause at adult stages, a low titer or absence of juvenile

hormone (JH) in the hemolymph is crucial for diapause induction (Nation, 2015). It is known in several species that JH production rates are changed by photoperiod. For example, in adults of the Colorado potato beetle *Leptinotersa decemlineata* and the mosquito *Culex pipiens*, the JH biosynthetic activities of the CA under long-day conditions are higher than those under short-day conditions (Khan et al., 1982; Readio et al., 1999). Therefore, to clarify how the brain controls endocrine effectors in photoperiodic responses, JH is appropriate as a target hormone.

JHs represent a family of acyclic sesquiterpenoids. They are principal products of the corpora allata (CA) which are retrocerebral glands of ectodermal origin (Wigglesworth, 1970). JHs have been identified in many insect species. Wigglesworth has initially observed that JHs are necessary for growth and development in immature insects, whereas we now know JHs are involved in other physiological functions, such as caste determination, locomotor behavior, stress response, and diapause (Nijhout, 2003). Röller and his collegues first identified the principal JH in lipid extracts of *Hyalophora cecropia* (Röller et al., 1967). This JH has been first identified as methyl (*2E,6E 10-cis*)-10,11-epoxy-7- ethyl-3,11-dimethyl-2,6- tridecadienoate, and termed JH I. Since that time, more than 10 types of JHs, such as JH II, JH III, and JHB3, have been identified as naturally occurring JHs. JH III appears to be the most common JH among the species studied (Goodman and Cusson, 2012). Identification of JH structures has allowed us developing a radiochemical assay for the direct measurement of JH production by the CA (Pratt and Tobe, 1974; Tobe and Pratt, 1974).

Molecules controlling JH biosynthesis, stimulatory peptides called allatotropins (ATs) and

inhibitory peptides called allatostatins (ASTs), have been identified in several species (Williams, 1961; Girardie, 1966; Tobe, 1980). Biological amines such as octopamine, dopamine, and serotonin (5-hydroxytryptamine) are also reported to effect on JH biosynthesis (Thompson et al., 1990; Rachinsky, 1994; Granger et al., 1996). Neurons innervating the CA have been identified with somata in the pars intercerebralis (PI) and pars lateralis (PL) of the protocerebrum. In the cockroach *Diploptera punctata*, the PL somata innervating the CA by way of the nervi corporis cardiaci (NCC) are AST-immunoreactive (Stay et al., 1992). In the crickets *Gryllus bimaculatus* and *Acheta domesticus*, somata of the PL and PI, and NCCs are AST-immunoreactive (Neuhäuser et al., 1994). These results indicate that ASTs affect the CA through NCCs from the PL or PI. However, *D. punctata*, *G. bimaculatus* and *A. domesticus* do not have photoperiodism and it is not known that these molecules and neurons are involved in photoperiodic control of JH biosynthesis. In insects which have reproductive diapause, interestingly the PI and/or PL are involved in diapause control. Ablation experiments have revealed that development of the reproductive organs are inhibited by the PL and promoted by the PI in several insects showing reproductive diapause (Hodková, 1976, 1979; Poras, 1982; Poras et al., 1983; Shiga and Numata, 2000; Shimokawa et al., 2008). However, it is totally unknown whether these regions control JH biosynthesis. Only in *L. decemlineata* and the migratory locust *Locusta migratoria*, cauterization experiments suggest that the PL plays an inhibitory role in JH biosynthesis by the CA under diapause conditions (Khan et al., 1986; Poras et al., 1983). In these insects, however, regulatory molecules and neurons of JH biosynthesis have not been identified.

The brown-winged green bug *Plautia stali* shows clear photoperiodic regulation of reproductive diapause (Kotaki and Yagi, 1989). Kotaki (1999) showed that biosynthetic activities of the CA products under long-day conditions (light:dark [L:D] = 16:8 h) at high temperature (25°C) were higher than those under short-day conditions (L:D = 12:12 h) at low temperature (20°C). Later the CA products have been identified as methyl (*2R,3S,10R*)-2,3;10,11-bisepoxyfarnesoate in *P. stali* (Kotaki et al., 2009). This was a novel JH known as ‘‘juvenile hormone III skipped bisepoxide ($JHSB_3$)” first identified in heteropteran insects. In CA-removed or diapause adults, $JHSB_3$ application stimulated development of ovaries in females and ectadenia in males (Kotaki et al., 2011). The result suggests that JH is a sole endocrine hormone photoperiodically regulating reproductive organs in *P. stali*. Therefore, I thought *P. stali* is a good model species for analysis of neuroendocrine mechanisms underlying photoperiodic responses. Also, identification of the JH structure of this species has made it more reliable to determine JH biosynthetic activity by using radiochemical assay.

Here I investigated how the brain regulates JH biosynthesis under different photoperiodic conditions in *P. stali* and regulatory molecules of JH biosynthesis in the brain to discuss neuroendocrine mechanisms underlying photoperiodism. In Chapter 1, I examined whether JH biosynthetic activities are photoperiodically controlled in *P. stali*, and roles of the brain in JH biosynthesis. Moreover, I examined neurons in the brain innervating the CC-CA. In Chapter 2, I characterized and identified allatoregulatory molecules in the brain of *P. stali* to discuss how the brain photoperiodically regulates JH biosynthesis.

Abbreviations

ABC	avidin-biotin complex
Apime-AST	C-type allatostatin of *Apis mellifera*
AST	allatostatin
AT	allatotropin
CA	corpus allatum
CC	corpus cardiacum
Dippu-AST	A-type allatostatin of *Diploptera punctata*
FM	flight muscle
GnRH	gonadotropin-releasing hormone
Grybi-MIP/AST	B-type allatostatin of *Gryllus bimaculatus*
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IQR	interquartile range
JH	juvenile hormone
$JHSB_3$	juvenile hormone III skipped bisepoxide
mBr	brain expect for the optic lobe
NCC	nervi corporis cardiaci
OL	optic lobe
Peram-AT	allatotropin of *Periplaneta americana*
PI	pars intercerebralis
PL	pars lateralis
PL-d	dorsal-pars lateralis
PL-v	ventral-pars lateralis
Plast-MIP/AST	B-type allatostatin of *Plautia stali*
Plast-sNPF	short-neuropeptide F of *Plautia stali*
RpL32	ribosomal protein L32
PTTH	prothoracicotropic hormone
SEG	subesophageal ganglion
sNPF	short neuropeptide F
TLC	thin-layer chromatography

Chapter 1

Role of the brain in photoperiodic regulation of juvenile hormone biosynthesis in *Plautia stali*

Abstract

I investigated the roles of the brain in photoperiodic regulation of juvenile hormone (JH) biosynthesis in *Plautia stali* (Heteroptera: Pentatomidae). Within 4 days after transferring the adults from short-day to long-day conditions, the biosynthesis of juvenile hormone III skipped bisepoxide became higher than that under short-day conditions. When the corpus cardiacum-corpus allatum complex (CC-CA) was coincubated with different regions of the brain, JH biosynthetic activities were inhibited by a middle part of the brain, i.e., the brain excluding the optic lobes, extirpated from short-day and long-day females. Backfills from the CC-CA revealed 3 groups of somata in the middle part of the brain: 15-18 pairs of somata were stained in the pars intercerebralis, 9-10 pairs were stained in the pars lateralis, and 5 pairs were stained on the nervi corporis cardiaci 2. These results suggest that inhibitory molecules of JH biosynthesis are produced in the middle part of the brain, regardless of photoperiod, but their release is suppressed under long-day conditions, leading to an increase in JH biosynthetic activities. Further, some neurons with somata in the pars intercerebralis, in the pars lateralis, or on the nervi corporis cardiaci 2 might be involved in photoperiodic regulation of JH biosynthesis.

Introduction

Many multivoltine insects living in temperate regions anticipate seasonal changes by photoperiod and enter diapause during unfavorable seasons. Diapause is a developmental arrest accompanied by major shutdown of metabolic activity and a genetically programmed response that occurs at species-specific stages of eggs, larvae, pupae, or adults (Denlinger et al., 2011). Gonadal development is inhibited in insects entering diapause at the adult stage. Juvenile hormones (JHs), biosynthesized and secreted by the corpus allatum (CA), are required for gonadal development. However, in many insects, JH biosynthetic activities from diapause adults are known to be lower than those under non-diapause conditions (Denlinger et al., 2011), and neural and hormonal mechanisms of photoperiodic regulation of JH biosynthesis have not been understood thus far.

In the viviparous cockroach *Diploptera punctata*, the two-spotted cricket *Gryllus bimaculatus*, and the tobacco hornworm *Manduca sexta*, allatostatins or allatotropins are known as regulatory neuropeptides of JH biosynthesis (Kramer et al., 1991; Lorenz et al., 1995; Woodhead et al., 1989). However, in these insects, JH biosynthesis is not photoperiodically controlled and the mechanism by which environmental signals control JH biosynthetic activities remain unknown. Under diapause conditions, transections of the nerve between the brain and CA increase JH biosynthetic activities in the Colorado potato beetle *Leptinotarsa decemlineata* (Khan et al., 1983). Extracts of the brain-corpus cardiacum (CC) complexes from diapause

females inhibit JH biosynthetic activities in the migratory locust *Locusta migratoria* (Okuda and Tanaka, 1997). These results suggest that JH biosynthesis by the CA is regulated by neural factors and some molecule produced in the brain or CC.

In some insects, brain neurons innervating the CA have somata in regions called the pars intercerebralis (PI) and pars lateralis (PL), where different kinds of neurosecretory cells are located (Raabe, 1989). With an interest in involvement of the CA in the control of ovarian development, roles of the PI and PL in reproduction and adult diapause have been examined in several species. In *L. migratoria* and the bean bug *Riptortus pedestris*, neurons in the PL inhibit vitellogenesis during diapause (Poras et al., 1983; Shimokawa et al., 2008). In the blowfly *Protophormia terraenovae*, the PL suppresses ovarian development under diapause conditions, while the PI promotes it under non-diapause conditions (Shiga and Numata, 2000). In the linden bug *Pyrrhocoris apterus* and the common groundhopper *Tetrix undulata*, neurons in the PI are necessary to suppress ovarian development during diapause (Hodková, 1976, 1979; Poras, 1982). These results show that the PI and PL play important roles in the regulation of adult diapause. However, it is little known whether these regions regulate JH biosynthesis to change reproductive states. Only cauterization experiments in *L. decemlineata* and *L. migratoria* suggest that the PL plays an inhibitory role in JH biosynthesis by the CA under diapause conditions (Khan et al., 1986; Poras et al., 1983). In these insects, however, regulatory neurons of JH biosynthesis have not been identified. Identification of brain neurons projecting to the CC-CA and brain regions important for the control of JH biosynthesis could be the first step in

the investigation of regulatory neurons of JH biosynthesis. It is also important to use an insect species that shows clear photoperiodic regulation of JH production.

The brown-winged green bug *Plautia stali* shows clear photoperiodic regulation of reproductive diapause (Kotaki and Yagi, 1989). Before identification of the CA product in *P. stali*, in combination with temperature, Kotaki (1999) showed that biosynthetic activities of the CA products under long-day conditions (light:dark [L:D] = 16:8 h) at high temperature (25°C) were higher than those under short-day conditions (L:D = 12:12 h) at low temperature (20°C). Therefore, it is highly possible that *P. stali* shows clear photoperiodic regulation of JH biosynthesis. Although JHs have been found as various kinds of acyclic sesquiterpenoids in different insect species, JH structures in heteropteran insects had not been understood until methyl (2R,3S,10R)-2,3;10,11- bisepoxyfarnesoate, known as "juvenile hormone III skipped bisepoxide ($JHSB_3$)," was found as a novel JH in *P. stali* (Kotaki et al., 2009). Identification of the JH structure has made it possible to determine JH biosynthetic activity in *P. stali*.

In the present study, I compared $JHSB_3$ biosynthetic activities by radiochemical assay and ovarian development in *P. stali* under long-day and short-day conditions at a fixed temperature. For understanding the roles of the brain in JH biosynthesis, I measured the JH biosynthetic activities of the CA coincubated with different regions of the brain. To find plausible neurons regulating JH biosynthesis by the CA, I examined the neurons in the cephalic ganglia innervating the CC or CA.

Materials and Methods

Insects

A stock culture of *P. stali*, maintained for more than 30 generations under long-day conditions (L:D = 16:8 h) at 25°C at the National Institute of Agrobiological Sciences (Tsukuba, Japan), was used. Insects were reared on dry soybeans, raw peanuts, and water supplemented with 0.05% sodium L-ascorbate and 0.025% L-cysteine (Kotaki and Yagi, 1987). Diapause insects were obtained by rearing larvae and adults under short-day conditions (L:D = 12:12 h) at 25 ± 1°C. To examine photoperiodic control of JH biosynthetic activities and ovarian development, females individually kept under short-day conditions for 15 days after adult emergence (day 15) were kept under the same conditions or transferred to long-day conditions.

Ovarian stages

After females were dissected in 0.9% NaCl, vitellogenesis in oocytes was examined under a stereoscopic microscope and the minor diameter of the terminal oocyte was measured (NIS-Elements BR 3.0; Nikon, Tokyo, Japan). Classification of ovarian stages was adopted from Morita and Numata (1999): (I) no vitellogenic oocyte, (II) vitellogenic oocytes with a minor diameter of < 0.4 mm, and (III) vitellogenic oocytes with a minor diameter of ≥ 0.4 mm.

Females with stage (I) ovaries were in diapause and those with stage (II) and (III) ovaries were non-diapause females.

Radiochemical assay in vitro for JH biosynthetic activity

JH biosynthetic activities of the CA were measured on days 15, 19, 21, 23, and 25. Methods for the radiochemical assay were adopted from Kotaki (1993). In brief, the minimum essential medium (with Hank's salt and L-glutamate and without sodium bicarbonate; GIBCO, Palo Alto, CA, USA) for the radiochemical assay was added with 20 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; SIGMA, St. Louis, MO, USA) and 5 ppm of Tween 80 (SIGMA) and adjusted to pH 7.2. To this medium, L-[methyl-^{3}H] methionine (Amersham; Buckinghamshire, UK; PerkinElmer; Branchburg, NJ, USA) was added to obtain a final specific activity of 14.7 MBq/mmol. For incubation of the CC-CA, siliconized (Siliconise L-25, Fuji Systems, Tokyo, Japan) glass vials (6 mm × 30 mm, Maruemu, Osaka, Japan) were used. After the CC-CA was incubated in 40 µL medium for 3 h at 30°C, the medium was extracted with 60 µl of hexane once. $JHSB_3$ and JHB_3 as lateral markers and 30 µL of the hexane extract were applied to different lanes in a silica thin-layer chromatography (TLC) sheet, Empore® (20 cm × 10 cm) (Analytichem International, Frampton Ave Harbor City, CA, USA). The TLC sheet was then developed with a 3:1 mixture of hexane-ethyl acetate. Spots of unlabeled standards were visualized with ultraviolet light and with phosphomolybdic acid solution with heat. The

remaining TLC sheet was cut into strips (5-mm long) and radioactivity on the same lane as $JHSB_3$ in the strips was measured with scintillation liquid (ASC II from Amersham; Ultima Gold from PerkinElmer) by using a liquid scintillation counter, LS6500 (Beckman Coulter, Fullerton, CA, USA).

Coincubation of the CC-CA with the brain

The brain, subesophageal ganglion (SEG), and CC-CA were derived from day 25 females reared under short-day conditions or day 25 females which were transferred to long-day conditions from short-day conditions at day 15. The CC-CA from a female under long-day conditions was coincubated with the brain-SEG complex (Br-SEG) from 3 females under long-day conditions or with the Br-SEG from 3 females under short-day conditions. The CC-CA from a female under short-day conditions was coincubated with the Br-SEG from 3 females under long-day conditions.

To examine the important regions of the cephalic ganglion for the control of JH biosynthesis, the cephalic ganglion was divided into 3 portions: the optic lobe, a middle part of the brain (mBr), and SEG (Fig. 1-1). The CC-CA from a female under long-day conditions was coincubated with the mBr, optic lobes and SEG (OL-SEG), or pieces of the flight muscle (FM) from 3 females under short-day conditions or with the mBr from 3 females under long-day conditions.

Staining of neurons projecting to the CC-CA

Adult females reared under long-day conditions for ≥10 days after adult emergence were used for neuronal staining. Methods for backfills and visualization of neurons were adopted from Shimokawa et al. (2008). In brief, females were mounted in clay. The tip of a small glass pipette was filled with 1% dextran tetramethylrhodamine and biotin (3 kDa; Molecular Probes, Eugene, OR, USA). The cut end of the aorta attached to the CC-CA was drawn into the dye-filled pipette. To stain neurons unilaterally, the nervus corporis cardiaci (NCC) was unilaterally severed before dye-fills (Fig. 1-1). To stain brain neurons projecting to the CC-CA partially, the neurons were severed selectively with a tungsten knife before backfills. Clay-mounted females with the dye-filled pipette were left in a moist chamber at room temperature. After 3-5 h of incubation, surviving females were processed according to the following steps. The brain with the SEG was dissected out in 0.9% NaCl solution and fixed in 4% paraformaldehyde for 24 h at 4°C. After washing with 0.1 M phosphate-buffered saline with 0.5% Triton X-100 (pH 7.2), the brain was dehydrated in an alcohol series and cleared in methyl salicylate. The whole-mount preparations were observed under a fluorescent microscope with a filter set for rhodamine (BX50-FLA; Olympus, Tokyo, Japan). A charge-coupled device camera (Digital Sight; Nikon, Tokyo, Japan) digitized the microscopic images.

After obtaining microphotographs, a fluorescent image was converted to a monochromatic image through the avidin-biotin complex (ABC) method in order to trace the neurons. The

preparations were observed under a microscope (BX50; Olympus), and stained neurons were traced using a drawing tube (BX50-UDA; Olympus). The size of somata was measured using the NIS-Elements BR software (Nikon). Fluorescent images and traces of neurons were processed for Adobe Photoshop CS5 (Adobe Systems Incorporated, San Jose, CA, USA) and Corel Draw 9.0 (Corel Corp., Ottawa, Ontario, Canada).

Results

Ovarian stages and JH biosynthetic activities

Under short-day conditions, on days 15, 19, 21, and 23, all the females had immature ovaries. On day 25, 87% females (N = 15) had immature ovaries (Fig. 1-2A). Under long-day conditions, 11% females (N = 18) already had vitellogenic ovaries on day 19 (Fig. 1-2A), and the proportion of females having vitellogenic ovaries increased gradually. All the females had mature ovaries on day 25. Thus, 10 days were sufficient for all the females to terminate diapause.

Under short-day conditions, JH biosynthetic activities were low throughout the experimental period (Fig. 1-2B), and on day 25, JH biosynthetic activities in 2 females with vitellogenic ovaries were higher than those in females with immature ovaries (data not shown). In contrast, under long-day conditions, ovaries developed as JH biosynthetic activities gradually increased. On days 19, 21, 23, and 25, JH biosynthetic activities under long-day conditions were significantly higher than those under short-day conditions.

Effects of the brain on JH biosynthetic activities

When the CC-CA from a long-day female was coincubated with the Br-SEG from long-day

females or with the Br-SEG from short-day females, the JH biosynthetic activities of the CA were significantly lower than those of the CA without the Br-SEG (Fig. 1-3A).

When the CC-CA from a short-day female was coincubated with the Br-SEG from long-day females, no significant difference was observed in JH biosynthetic activities, compared with those of the CC-CA alone (Fig. 1-3B). The Br-SEG from long-day females did not promote the JH biosynthetic activities of the CA from short-day females.

To examine the regions of the Br-SEG important for the suppression of JH biosynthetic activities, the CC-CA from a female under long-day conditions was coincubated with different parts of the cephalic ganglion. When the CC-CA was coincubated with the mBr from short-day females or with the mBr from long-day females, JH biosynthetic activities were significantly lower than those when the CC-CA was coincubated with OL-SEG or with FM (Fig. 1-4). There was no significant difference in the JH biosynthetic activities of the CC-CA alone, the CC-CA with FM, and the CC-CA with OL-SEG. Only the mBr inhibited the JH biosynthetic activities of the CA.

Anatomy of neurons innervating the CC-CA

Neurons projecting to the CC-CA were stained. Bilateral backfills from the CC-CA revealed 3 groups of somata (Figs. 1-5 and 1-6A). Because backfills did not stain all the somata in every fill, I examined 15 preparations and the maximum number of stained somata was determined for

each group of neurons (Table 1-1): 15-18 pairs of somata were stained in the PI, 9-10 pairs were stained in the PL, and 5 pairs were stained on the NCC2 in the brain. In the PI, the major diameter of the somata was 12.1 ± 4.5 μm (*N*= 13) and the size of the somata showed a large variation. Their axons exited to the CC-CA through the NCC1. These neurons were designated as PI neurons according to the location of the somata. Two clusters of somata were found in the PL of each hemisphere. One cluster was located dorsally and anteriorly to the other cluster: the former and latter were designated as PL-d and PL-v, respectively. The PL-d had 3 somata (major diameter, 5.8 ± 2.5 μm; *N* = 13), and the PL-v had 6-7 somata (major diameter, 9.1 ± 2.2 μm; *N* = 13) (Table 1-1). All the somata in the PL clusters and on the NCC2 extended their axons to the CC-CA through the NCC2. Several fibers dorsomedially extended from the NCC2 to the PI. Five somata were stained on the NCC2 (major diameter, 6.2 ± 1.1 μm; *N* = 13).

For staining neurons projecting to the CC-CA though the NCC2, regions around the NCC1 were severed backward with a tungsten knife before backfills. Backfills from the CC-CA after severing the NCC1 stained somata in the PL (Fig. 1-6B). From the neurites of PL-d neurons, a few fibers extended to the PI and the others branched out in the ipsilateral NCC2. No somata were stained in the PI.

Unilateral backfills stained somata in the contralateral PI and the ipsilateral PL (Figs. 1-7 and 1-8). There were no ipsilateral axons of PI neurons or contralateral axons of PL neurons connected to the CC-CA. PL-d and PL-v neurons exited to the CC through the ipsilateral NCC2. A few fibers from PL-d or PL-v neurons were found running across the mid line.

Discussion

Although differences in the biosynthetic activities of some unknown products of the CA from diapause females and of those of the CA from reproductive females have been shown in the previous studies on heteropteran insects, the products synthesized by the CA had not been identified. On the basis of the identification of the CA products, like $JHSB_3$ in *P. stali* (Kotaki et al., 2009), I could show that in Heteroptera, $JHSB_3$ biosynthetic activity of the CA is significantly lower under short-day conditions than under long-day conditions.

JH biosynthetic activities remained low and no ovaries developed under short-day conditions, while the activities increased and ovaries developed in response to long-day conditions. Only 4 days after the transfer of the females from short-day to long-day conditions, JH biosynthetic activities became significantly higher than those under short-day conditions. This result shows that 4 cycles of photoperiod are enough to change JH biosynthetic activities in *P. stali*. This is a quick and clear photoperiodic response and favorable for investigating the neuroendocrine mechanism of photoperiodism. In the present study, an increase in JH biosynthetic activities occurred before yolk deposition in the oocytes. Kotaki et al. (2011) showed that $JHSB_3$ application to non-diapause adults with the CA ablated or to diapause adults induces the development of reproductive organs. This indicates that increase in $JHSB_3$ biosynthesis induces ovarian development under long-day conditions and that low $JHSB_3$ biosynthetic activities result in the suppression of ovarian development under short-day

conditions in *P. stali*.

The present study revealed that the mBr exerts inhibitory effects on JH biosynthetic activities, while the mBr does not promote the activities. These results suggest that JH biosynthesis is controlled mainly by allatostatic molecules produced in the mBr. Further, the allatostatic molecules are produced regardless of photoperiodic conditions, indicating that their release is suppressed under long-day conditions to increase JH biosynthetic activities. The allatostatic molecules from the mBr possibly seep into the medium during coincubation with the CA, from the cut end of the NCC.

This inhibitory effect is in contrast to the effects observed in the linden bug *Pyrrhocoris apterus*. In *P. apterus*, the total extracts of the brain, SEG, CC, and CA, under diapause and non-diapause conditions, exert stimulatory effects on the biosynthetic activity of the CA, suggesting the existence of allatotropic molecules in the bug (Hodková et al., 1996). In both *P. stali* and *P. apterus*, regulatory molecules of JH biosynthesis seem to exist regardless of photoperiodic conditions, and their secretion is probably controlled by photoperiod. In *P. stali*, the secretion of allatostatic molecules may begin to be inhibited in 4 days from the transfer of adults to long-day conditions, following which JH biosynthetic activity gradually increases.

The present study revealed that 3 kinds of neurons with somata in the PI, in the PL, and on the NCC2 project to the CC-CA. Fibers from PI and PL neurons interlace each other in a region between PI and PL somata. Morphological features suggest that PI and PL neurons have connections in the protocerebrum. PL neurons projected to the CC-CA only ipsilaterally and

were classified into 2 groups in *P. stali*: PL-v and PL-d. In *M. sexta*, the silkworm *Bombyx mori*, *P. terraenovae, L. migratoria*, the cricket *Teleogryllus commodus*, and *R. pedestris*, in which the projection patterns of PL neurons have been examined by unilateral backfills, 2 types of PL neurons have been reported (Copenhaver and Truman, 1986; Ichikawa, 1991; Shiga et al., 2000; Shimokawa et al., 2008; Virant-Doberlet et al., 1994). In these species, except *R. pedestris*, one type sends ipsilateral axons and the other sends contralateral axons to the retrocerebral complex. There are usually 2 pairs of PL neurons with contralateral axons. However, in *R. pedestris* and *P. stali*, no contralateral somata in the PL were observed by unilateral backfills. Absence of PL neurons with contralateral projections is unique to these 2 species, although the functional significance of ipsilateral and contralateral projection patterns has not been determined for any species.

Somata in the PI, in the PL, and on the NCC2 were located in the mBr, which exerted inhibitory effects on JH biosynthesis. In a previous study, NCC transection between the brain and CC promoted JH biosynthesis slowly even under diapause-maintaining conditions (Kotaki, 1999). Considering that the neurons with somata in the PI, in the PL, and on the NCC2 have axons in the NCC, it is probable that some of these neurons produce allatostatic molecules. In the adults of *D. punctata*, allatostatins are produced in PL neurons projecting to the CC-CA (Stay et al., 1992). In *P. stali* too, allatostatic molecules might exist in the PL.

In *D. punctata*, allatostatins are released directly within the CA and then into the hemolymph from the CA for inhibiting JH biosynthesis (Lloyd et al., 2000; Stay et al., 1995). In

B. mori, it is considered that an allatotropin promotes JH biosynthesis through inhibition of release of short neuropeptide F (sNPF), since allatotropin receptors exist in CC cells producing sNPF (Yamanaka et al., 2008). In *P. stali* too, there are both possibilities that neurons projecting to the CA directly regulate JH biosynthesis and that neurons projecting to the CC regulate JH biosynthesis in a paracrine fashion or through other neurons. Next, it is necessary to identify allatostatic molecules in *P. stali* in order to investigate the neurons that photoperiodically control JH biosynthesis by the CA.

Chapter 2

Regulatory molecules of juvenile hormone biosynthesis in *Plautia stali*

Abstract

Allatostatic molecules which inhibit JH biosynthesis were characterized and identified in the brown-winged green bug *Plautia stali*. Methanol extracts of the brain showed a strong inhibition of JH biosynthesis. Allatostatic effects were found in any part of the cephalic ganglia, but not in the fat body or muscle tissues. The allatostatic activities of the brain extracts were not affected by heat treatment but gently suppressed by trypsin treatment. Seven peptides and 3 biogenic amines which are known to have allatoregulatory functions in other species were examined on the allatostatic activities in *P. stali*. Among these peptides and amines, only B-type allatostatin (AST), AWRDLSGGW-NH_2, found in *Gryllus bimaculatus* inhibited JH biosynthesis of *P. stali*. I found a cDNA sequence encoding a peptide precursor of B-type ASTs named Plast-MIP/AST in *P. stali*. A synthetic peptide, AWKDLSKAW-NH_2, deduced from the precursor sequence showed clear inhibition of JH biosynthesis of *P. stali*. mRNA expression of the AST precursor was detected in the brain. Plast-MIP/AST is first identified as a functional allatostatin in hemipteran insects. Allatostatic effects were ubiquitous in the cephalic ganglia, suggesting that ASTs of *P. stali* have also other unknown functions in addition to allatostatic activities.

Introduction

Juvenile hormones (JHs), synthesized and released by the corpus allatum (CA), are required for growth regulation and reproduction in insects. Many multivoltine insects living in temperate regions anticipate seasonal changes by photoperiod and adjust their growth and reproduction to occur in favorable seasons. It has been reported that insect species control seasonal growth and reproduction by JH of which biosynthesis is changed by photoperiod (Denlinger et al., 2011). However, neural mechanisms underlying photoperiodic regulation of JH biosynthesis have not been understood.

JH inhibitory and stimulatory factors have been reported in several species. Neuropeptides, allatostatins (ASTs), short-neuropeptides F (sNPFs) and allatotropins (ATs), have been reported as regulatory factors of JH biosynthesis (Bendena and Tobe, 2012). ASTs comprise a peptide family classified into 3 types: A-type, neuropeptides with a consensus C-terminal sequence of Y/FXFGL-NH_2, B-type with a C-terminal sequence of $W(X)_6W$-NH_2, and C-type with a C-terminal sequence of PISCF, some of which are C-terminally-amidated. Since A-type ASTs were first identified from the cockroach *Diploptera punctata*, AST sequences have been found in many insect orders. However, inhibitory functions of JH biosynthesis have been demonstrated in rather small number of species such as the two-spotted cricket *Gryllus bimaculatus* and the tobacco hornworm *Manduca sexta*. In only the silk worm *Bombyx mori*, sNPFs and an AT inhibit JH biosynthesis (Yamanaka et al., 2008; Kaneko and Hiruma, 2015),

although ATs are known as stimulatory molecules in other insects (Bendena and Tobe, 2012). Also, octopamine, dopamine and serotonin inhibit or stimulate JH biosynthesis, and its regulatory functions depend on species or developmental stages within the same species (Goodman and Granger, 2005). However, it is not known that these molecules control JH biosynthesis under different photoperiod.

It was reported in the mosquito *Culex pipiens* that knockdown of mRNA levels of AT in females under non-diapause conditions resulted in a cessation of ovarian development akin to diapause. This arrest in ovarian development was reversed with an application of JH (Kang et al., 2014). Although these results suggest that AT expression is necessary for JH biosynthesis under long-day conditions, there is no evidence for direct connection between an AT and JH biosynthesis.

The brown-winged green bug *Plautia stali* shows clear photoperiodic regulation of reproductive diapause (Kotaki and Yagi, 1989). In the previous study, methyl (2R,3S,10R)-2,3;10,11-bisepoxyfarnesoate, known as ‘‘juvenile hormone III skipped bisepoxide ($JHSB_3$)’’, was found as a novel JH in *P. stali* (Kotaki et al., 2009). The JH biosynthetic activities of the CA also show a clear photoperiodic response (Chapter 1). Females reared under long-day conditions show higher activities of JH production than those under short-day conditions. A brain region including the pars lateralis (PL) and pars intercerebralis (PI) neurons, which innervate a complex of the corpus cardiacum (CC)-CA, inhibits JH biosynthetic activities by the CA from long-day conditions *in vitro* (Chapter 1). These results suggest that allatostatic factors

reside in the brain. Because there is no allatotropic activities in the brain, JH biosynthesis in *P. stali* is mainly regulated by allatostatic factors (Chapter 1). This allatostatic factor may mediate photoperiodic information to the CA for a control of JH productions. In the present study, I characterized and identified allatostatic molecules in the brain of *P. stali*.

Materials and Methods

Insects

The same stock culture of *P. stali* as in Chapter 1 was used. Insects were reared on raw peanuts and water supplemented with 0.05% sodium L-ascorbate and 0.025% L-cysteine. To prepare 80% methanol extracts of the cephalic ganglia, insects were obtained by rearing larvae and adults under short-day conditions (LD = 12:12 h) at 25±1°C. For the quantitative PCR, I used another strain, which show clear photoperiodic response, collected by a pheromone (Shin-Etsu Chemical, Tokyo, Japan) with light in Kawachinagano (34.27°N, 135.34°E), Japan.

Extraction of the cephalic ganglia

Tissues of the cephalic ganglia were obtained from females on day 25 (adopted to Chapter 1) under short-day conditions. The cephalic ganglia were extirpated and divided into the middle part of the brain (mBr), optic lobe (OL) and subesophageal gangalion (SEG) using a sharpened tungsten knife in 0.9% NaCl. The mBr was further divided into the pars intercerebralis (PI), pars lateralis (PL) and ventral mBr regions (Fig. 2-1). Seven kinds of brain tissues were prepared. Brain tissues from 11 females were pooled in a 1.5 mL tube and homogenized in 200 μL of 80% methanol. As controls, the flight muscle or fat body from 2-3 females (roughly the same volume

of the mBr from 11 females) was also homogenized in 80% methanol. After centrifugation (10,000 g, 10 min, 4°C), the supernatant was transferred to a new 1.5 mL tube and dried under reduced pressure. The extracts were stored at -80°C.

Heat-treatment and Trypsin-treatment of the mBr extract

For the heat-treatment experiment, the mBr (shown in figure 2-1) from 11 females were put into 40 µL distilled water in a 1.5 mL tube and then the tube was put into boiling water for 15 min. After the treatment, 160 µL methanol was added to the tube and homogenized. After centrifugation (10,000 g, 10 min, 4°C), the supernatant was transferred to a new tube and dried under reduced pressure. The extract was stored at -80°C before experiments.

For the trypsin-treatment, the brain extract was prepared as described in the above section. I dissolved 1 mg trypsin (porcine pancreas, 4100 USP Trypsin Units/mg, Wako Pure Chemical Industries, Osaka, Japan) in 1 mL 50 mM Tris-HCl buffer (pH 7.8) with 10 mM $CaCl_2$ (Kilby and Youatt, 1954). The extract was dissolved in 11 µL of the trypsin solution or the Tris-HCl buffer alone. The extract in the trypsin solution was incubated at 37°C for 16 h and then incubated at 100°C for 30 min to inactivate trypsin. The Tris-HCl alone, the trypsin solution alone, or the brain extract in the Tris-HCl was also incubated at 37°C for 16 h and then incubated at 100°C for 30 min.

Preparation of peptides and amines

To examine allatoreguratory effects, 7 peptides and 3 amines were used. Sequences of the peptides are shown in Table 2-1. Dippu-AST2 (Pratt et al., 1991) (SIGMA, St. Louis, MO, USA), Dippu-AST8, Dippu-AST9 (Woodhead et al., 1989) (SIGMA) were commercially available. Grybi-MIP/AST1 (Wang et al., 2004), Plast-MIP/AST1 (Tanaka and Shinoda, unpublished), Apime-AST (Hummon et al., 2006), Peram-AT (Neupert et al., 2009), Plast-sNPF (Tanaka and Shinoda, unpublished) were synthesized (Funakoshi, Tokyo, Japan). We adopted nomenclature of these peptides proposed by Coast and Schooley (2011). Octopamine, dopamine, serotonin were also commercially available (Sigma-Aldrich, Dorset, UK). These peptides or amines were added to the cold medium to make an undiluted solution. Then, the peptide or amine solution was diluted to the final concentrations with the radio-labeled (hot) medium. The peptide sequences and the final concentrations of the peptides and amines were shown in Table 2-1.

JH radiochemical assay

For measurement of JH biosynthetic activities, the CC-CAs were derived from day 25 females which were transferred to long-day conditions from short-day conditions at day 15. The methods for the radiochemical assay were adopted from Chapter 1. I used cintillation liquid, Ultima Gold (PerkinElmer, Branchburg, NJ, USA) and liquid scintillation counter,

Tri-Carb2910TR (PerkinElmer). To examine effects of the extracts of the cephalic ganglion tissues, heat-treated extract of the mBr, synthetic peptides, and amines, the JH biosynthetic activities were measured with a CC-CA in a serial 3 times incubation by the radiochemical assay. Each incubation was made for 3 h. In the first incubation the JH biosynthetic activities were measured in the hot MEM (see Chapter 1) without the extracts, peptides or amines. The dried extracts of the mBr, PI, PL, ventral mBr, OL, or SEG, each from 11 females, and flight muscle, or fat body roughly the same volume of the mBr from 11 females, were dissolved in 220μL of the hot medium and 40 μL (2 female equivalents) of the hot medium with the extracts was used for the second incubation. After the first incubation, the CC-CA was transferred to the second incubation medium with the extracts. In the third measurement again, the JH biosynthetic activities were assayed in the hot medium without the extracts. The percentage inhibition was calculated as a following equation.

$$\text{Percentage inhibition} = \left(1 - \frac{2 \times \text{2nd activity}}{\text{1st activity} + \text{3rd activity}}\right) \times 100$$

To examine effects of the trypsin-treated extract, the JH biosynthetic activities were measured by single incubations. The CC-CA was incubated in the hot medium for 3 h with the trypsin-treated extract (0.5 female equivalents) after pre-incubation for 3 h in the cold medium.

***Quantitative real-time PCR for* Plast-Mip/Ast**

The mBr derived from day 25 females which were transferred to long-day conditions from short-day conditions at day 15 and that from day 25 females continuously reared under short-day conditions were used. Total RNAs were isolated from the mBr (Fig. 2-1) of a female by using Trizol (Invitrogen, Gaithersburg, MD, USA) and a PureLink RNA extraction kit (Invitrogen). cDNAs were synthesized from the RNA samples by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). For real-time PCR analysis, 2.5% of the cDNA was used in a final concentration of 1xPower SYBR Green PCR Master Mix (Applied Biosystems) and 0.032 μM of each primer using 7500 Real-time PCR System (Applied Biosystems) according to the supplier's instructions. Each reaction was done in duplicate. Primers were Ast-F and Ast-R for *Plast-Mip/Ast*, and RpL32-F and RpL32-R for *ribosomal protein 32* (*RpL32*). Each primer set was designed to amplify an amplicon of approximately 100 bp. The nucleotide sequences of primers are shown in Table 2-2. Quantification of cDNAs was performed by the standard curve methodology. *RpL32* was used as a control gene for normalization. The quantification was made for each mBr, and 5 samples were measured for each photoperiodic condition.

Results

Effects of cephalic ganglia extracts on JH biosynthetic activities

When the CC-CA was incubated 3 times consecutively without extracts, the JH biosynthetic activities little increased especially from the 1st to 2nd incubation (Fig. 2-2, control). When the CC-CA was incubated with the extracts of mBr, strong inhibition was observed in the 2nd incubation. But the synthetic activities were recovered in the 3rd incubation. This inhibition was observed in any part of the cephalic ganglia (Fig. 2-3). A median of percentage inhibition in the intact group was -14.3% ［interquartile range (IQR), -53.8 to 3.5%］. When the CC-CA was incubated with extracts of the mBr, PL, PI, ventral mBr, OL or SEG, medians of percentage inhibition were 92.5% (IQR, 87.7 to 95.8%), 91.3% (IQR, 86.5 to 97.3%), 80.3% (IQR, 61.5 to 89.1%), 88.8% (IQR, 84.3 to 100%), 75.2% (IQR, 62.1 to 79.5%) and 95.9% (90.6 to 98.6%), respectively. However, when the CC-CA was incubated with extracts of the flight muscle or fat body, medians of percentage inhibition were -33.3% (IQR, -79.6 to -6.2) and -33.6% (IQR, -48.7 to -15.7%), respectively. The percentage inhibition of all parts of cephalic ganglia was significantly lower than the control, flight muscles and fat bodies.

Effects of heat treatment and trypsin treatment on allatostatic activities of the mBr extracts

To investigate physicochemical characteristics of allatostatic molecules in the mBr, its extract was treated by heat or with trypsin. Even after heat treatment, inhibition of JH biosynthesis occurred and percent inhibitions of the extract were not significantly different from those of the extract without heat-treatment (Fig. 2-4, Steel-Dwass test, $P > 0.05$).

Next, it was examined whether inhibitory effects by the mBr extracts were canceled by the trypsin treatment. When the CC-CA was incubated in the medium with the trypsin-treated extract, JH biosynthetic activities were significantly higher than those with the extract without trypsin treatment (Fig. 2-5, Steel-Dwass test, $P < 0.05$). Although the activities with trypsin-treated extracts appeared to be lower than those with normal medium and inactivated trypsin, there was no significant difference (Fig. 2-5).

Effects of peptides or amines on JH biosynthetic activities

To find allatostatic molecules in the brain, I examined effects of 7 peptides and 3 amines which have been reported to have allatoregulatory functions in other species on JH biosynthesis in *P. stali*. When the CC-CA was incubated with Dippu-AST2, Dippu-AST8, Dippu-AST9, Apime-AST, Peram-AST, Plast-sNPF, octopamine, dopamine, or serotonin (Table 2-1), the JH biosynthetic activities were not significantly different from those of the CC-CA incubated alone

(data not shown). However, only when the CC-CA was incubated with a B-type AST, Grybi-MIP/AST1, the JH biosynthetic activities were notably low (Fig. 2-6). The percentage inhibition at 1.0×10^{-6} M Grybi-MIP/AST1 (89.4%, IQR: 72.0 to 99.2%) was significantly higher than that at 1.0×10^{-8} M Grybi-MIP/AST1 (45.6%, IQR: 6.2 to 52.3%) and that in control (19.0%, IQR: -62.1 to 33.5%) (Steel-Dwass test, $P < 0.05$).

Sequence of* Plast-Mip/Ast *in* P. stali *and its effects on JH biosynthesis

Because a B-type AST in *G. bimaculatus* inhibited JH biosynthesis of *P. stali*, I searched cDNA sequences encoding B-type AST in *P. stali* from RNA sequencing (RNA-seq) data (Fig. 2-7). The deduced precursor peptide is 239 amino acids (Fig. 2-7) which contain potential cleavage sites producing 11 ASTs. Each AST was flanked by dibasic endoproteolytic cleavage sites K-R or K-K. These are preceded by a single G that is the signal for C-terminal amidation of ASTs by peptidyl-glycine α-amidation monooxygenase (Bradbury et al., 1982). I named each AST fragment Plast-MIP/AST1-9. Plast-MIP/AST4 and 5 duplicate in the precursor. Among 9 peptides, I examined effects of synthesized Plast-MIP/AST1 on JH biosynthesis. When the CC-CA was incubated with Plast-MIP/AST1 (concentration: 1.0×10^{-6} M and 1.0×10^{-4} M), the JH biosynthetic activities were considerably low (Fig. 2-8). The median of percentage inhibition of the control was 26.7% (IQR, -15.5 to 41.9%). With Plast-MIP/AST1, medians of percent inhibition were 35.9% (IQR, 8.1 to 59.6%) at 1.0×10^{-8} M, 97.2% (IQR, 92.9 to 100%) at $1.0 \times$

10^{-6} M and 93.9% (IQR, 91.6 to 98.2%) at 1.0×10^{-4} M. At concentrations of 1.0×10^{-6} M and 1.0×10^{-4} M Plast-MIP/AST1 significantly inhibited JH biosynthesis (Steel-Dwass test, $P < 0.05$).

Expression of mRNA encoding Plast-MIP/AST precursor in the mBr

Plast-MIP/AST precursor mRNA was detected in the mBr, and its expression levels in the mBr were not significantly different between short-day and long-day conditions (Mann-Whitney U-test, $P > 0.05$).

Discussion

The present study revealed that the inhibitory molecules in the mBr extract are heat stable but vulnerable by trypsin-treatment. The result suggests that allatostatic molecules in the mBr are peptides including K or R residues. This result fits quite well to the fact that Plast-MIP/AST1, AWKDLSKAW-NH_2, found as an inhibitory peptide in the present study has K residues. Moreover, gene expression of *Plast-Mip/Ast* was detected in the mBr. Therefore, it is probable that the inhibitory effects by the mBr on JH biosynthesis are due to Plast-MIP/AST produced in the mBr.

ASTs have been shown in Coleoptera, Dictyoptera, Orthoptera, Lepidoptera but not in Hemiptera. The present study first demonstrated an allatostatic peptide of AST families in hemipteran insects. Because Dippu-ASTs (A-type), a Apime-AST (C-type), a Peram-AT, a Plast-sNPF, octopamine, dopamine, and serotonin, which have been shown as allatostatic functions in other species, did not affect JH biosynthesis in *P. stali*, it is suggested that Plast-MIP/ASTs (B-type) is the main allatostatic molecule in *P. stali*.

The precursor peptide found in the present study contains 9 deduced ASTs, Plast-MIP/AST1-9. Plast-MIP/AST1, 2, 7, 8, and 9 are W$(X)_6$W-NH_2, and Plast-MIP/AST4-6 are W$(X)_7$W-NH_2. It has been reported that, B-type ASTs are usually W$(X)_6$W-NH_2, and only hemipteran insects, the pea aphid *Acyrthosiphon pisum* and the blood-sucking bug *Rhodnius prolixus*, have 2 types of W$(X)_6$W-NH_2 and W$(X)_7$W-NH_2 (Bendena and Tobe, 2012). Amino

acid sequences of Plast-MIP/ASTs agree to this. In *G. bimaculatus*, 4 Grybi-MIP/ASTs are deduced from cDNA sequence of an AST precursor (Wang et al., 2004) and 3 of them have been reported to inhibit JH biosynthesis (Lorenz et al., 1995). In *D. punctata*, 7 out of 13 Dippu-ASTs contained in a precursor peptide have allatostatic functions (Donly et al., 1993; Woodhead et al., 1989, 1993; Pratt et al., 1991). These results suggest that in *P. stali* not only Plast-MIP/AST1 but some other Plast-MIP/ASTs might have also allatostatic functions. Allatostatic activities of the mBr became weak after trypsin treatment but there seemed to be some inhibition. This suggests that trypsin treatment did not completely abolish allatostatic activities. Among Plast-MIP/AST1-9, only Plast-MIP/AST1, 8 and 9 contain K or R residues, indicating that trypsin does not degrade other 4 Plast-MIP/ASTs, and some of them might have allatostatic activities. In *G. bimaculatus*, Grybi-MIP/ASTs inhibit JH biosynthesis in a sigmoidal dose-dependent manner, and inhibitory effects increase abruptly from 10^{-8} M to 10^{-6} M (Lorenz et al., 1995). Critical concentrations of MIP/ASTs for allatostatic activities seem similar between *P. stali* and *G. bimaculatus*.

In insects showing photoperiodic regulation of JH biosynthesis, extracts of the central nervous system showed allatotropic effects in the linden bug *Pyrrhocoris apterus* and allatostatic effects in *L. migratoria* (Hodkova et al., 1996; Okuda and Tanaka, 1997). In these insects, however, allatoregulatory molecules have not been identified. In the mosquito *Culex pipiens*, JH biosynthetic activities of adult females increased rapidly after transferring to non-diapause conditions (LD = 16:8 h at 26°C) from diapause conditions (LD = 8:16 h at 15°C)

which inhibit JH biosynthesis (Readio et al., 1999). In *C. pipiens*, knockdown of AT mRNA using RNA interference in females reared under non-diapause conditions (LD = 15:9 h at 25°C) resulted in a cessation of ovarian development. The cessation of ovarian development could be reversed with an application of JH (Kang et al., 2014). These results indicate that AT promotes JH biosynthesis in *C. pipiens*. But, it remains unknown whether AT affects JH biosynthesis by the CA directly or indirectly because JH biosynthetic activities were not measured in the study. In the present study, an allatostatic molecule was found in *P. stali* which shows a photoperiodic control of JH biosynthesis. To examine whether Plast-MIP/ASTs are involved in neuroendocrine mechanisms for photoperiodic response, it is necessary to compare gene expression of *Plast-Mip/Ast* in the brain or its peptide contents in the hemolymph between different photoperiodic conditions.

I compared mRNA levels of *Plast-Mip/Ast* between short-day and long-day conditions. But no difference was observed. The result agrees to my previous study that allatostatic molecules are present in the mBr from both short-day and long-day females (Chapter 1). In females of *G. bimaculatus*, gene expression and immunoreactivity of B-type ASTs are observed in cells of not only the PI and PL innervating the CA but also of the SEG and tritocerebrum. This indicates that B-type ASTs have pleiotropic functions in *G. bimaculatus* (Witek et al., 1999; Wang et al., 2004). In *P. stali*, extracts of any part of cephalic ganglia inhibited JH biosynthesis, suggesting that Plast-MIP/ASTs in the cephalic ganglia also have pleiotropic functions. Based on these results, I consider 2 possibilities on photoperiodic regulation of JH biosynthesis via

Plast-MIP/AST. First possibility is that Plast-MIP/ASTs are produced under both short-day and long-day conditions, but their secretion to the CA is neurally controlled by photoperiod. Second possibility is that Plast-MIP/ASTs in neurons innervating to the CA are produced only under short-day conditions and its production is inhibited under long day conditions. But different expression levels are hidden by Plast-MIP/ASTs which are distributed in many other neurons in the brain. To reveal how photoperiod controls JH biosynthesis, it is necessary to compare gene expression of *Ast* or AST peptide contents in neurons projecting to the CC-CA under different photoperiodic conditions. If the first possibility is the case, the peptide contents in neurons projecting to the CC-CA are not different but the contents in the CC-CA and/or in the hemolymph are different between short-day and long-day conditions. Quantification of Plast-MIP/ASTs in the CC-CA or in the hemolymph should be taken in the next study. In the cabbage army moth *Mamestra brassicae*, almost the same amount of the prothoracicotropic hormone (PTTH), which stimulates ecdysteroid production in the prothoracic gland, exists in the brain under both diapause (LD = 10:14 h at 23°C) and non-diapause conditions (LD = 14:10 h at 25°C). However, PTTH titers were very low under diapause conditions, but high under non-diapause conditions. The results suggest that not production but secretion of PTTH is regulated by photoperiod and temperature (Mizoguchi et al., 2013). In *P. stali* also it might be more likely that photoperiod controls release of ASTs but not their production.

General Discussion

The mechanism of photoperiodic response comprises a “photoreceptor” distinguishing light from dark, a “photoperiodic clock” measuring length of day or night, and an “endocrine effector” regulating hormonal signaling (Saunders, 2002). Although it is probable that output signals from the photoperiodic clocks turn endocrine effectors on or off to regulate hormonal releases for appropriate physiological states, it has not been clarified how the brain including the photoperiodic clock system control endocrine effectors. Here I have revealed that the endocrine organs of the CA is photoperiodically regulated by the brain via allatostatic functions (Chapter 1), and propose an idea that a neuropeptide Plast-MIP/AST in the brain is responsible for suppression of JH biosynthesis and its secretion is controlled by photoperiod (Chapter 2).

It has been reported that release of PTTH in the brain, stimulating ecdysteroid biosynthesis, is inhibited in diapause-inducing conditions in *M. brassicae* which enter diapause at a pupal stage (Mizoguchi et al, 2013). In the Japanese quail *Coturnix japonica*, short-day conditions inhibit release of gonadotropin-releasing hormone (GnRH) which promote gonadotropin production and release (Perera and Follet, 1992). The nerve terminals of GnRH producing neurons are in close proximity to the basal lamina under long-day conditions, but encased by the endfeet of glia under short-day conditions (Yamamura et al., 2004). The neurons can release GnRH into portal blood when the terminals contact pericapillary space such as the basal lamina. Djungarian hamster *Phodopus sungorus* and the Syrian hamster *Mesocricetus auratus* also show

photoperiodic regulation of the gonads. In *P. sungorus* and *M. auratus*, there was no photoperiodic change in GnRH-immunoreactive cell bodies and expression of mRNA encoding GnRH in the brain, respectively (Yellon, 1994; Blown et al., 2001). These results indicate that photoperiod regulates release of GnRH but not their production in vertebrates. Photoperiodic neuroendocrine mechanisms seem to be common between vertebrates and invertebrates. Not production but release of brain neurosecretory molecules (such as Plast-MIP/ASTs, PTTH and GnRH) regulating production of endocrine hormones (such as gonadotropins, JHs, ecdysteroids) are regulated by photoperiods.

In hemipteran insects, presence of ASTs has been reported in only two species, the *A. pisum* and *R. prolixus* (Huybrechts et al., 2010; Ons et al., 2009, 2011). However, there is no evidence that ASTs of these insects inhibit their JH biosynthesis. So, the present study is the first report of an AST inhibiting JH biosynthesis in hemipteran insects.

Identification of inhibitory molecules of JH biosynthesis enables us to identify neurons inhibiting JH biosynthesis by Plast-MIP/AST immunocytochemistry. Plast-MIP/AST immunoreactive neurons directly innervating the CA are candidate as allatostatic neurons. In *D. punctata* and *G. bimaculatus*, immunoreactive neurons of ASTs have been reported to have somata in the PI, PL, and other regions (Stay et al., 1992; Witek et al., 1999). In *D. punctata*, approximately 30 immunoreactive neurons of ASTs with somata in the PL project to the CC-CA. In contrast, backfills from the CC-CA to the brain revealed neurons with approximately 60 somata in the PL (Chiang et al., 1999). These studies suggest that about a half of neurons

projecting to the CC-CA contains ASTs to inhibit JH biosynthesis. In *P. stali*, backfills from the CC-CA revealed smaller numbers of neurons. There are 9-10 somata in the PL and 15-18 somata in the PI. Part of them is considered to inhibit JH biosynthesis in *P. stali*. Probably, it is possible that output signals from the photoperiodic clock system regulate JH production through the allatostatic neurons. Within 4 days after transferring adults to long-day conditions, JH biosynthesis became higher than that under short-day conditions, indicating that determination of diapause induction or aversion after day-length measurement and day-number counting by a photoperiodic clock occurs within 4 days. After counting less than 4 cycles of long days, a photoperiodic clock might signal to stop releasing ASTs. In future, neural connections between plausible photoperiodic clock neurons and allatostatic neurons should be revealed to address a question how photoperiodic clocks regulate neuroendocrine systems.

Acknowledgements

I am grateful to Dr. Sakiko Shiga and Dr. Shin G. Goto, Osaka City University, and Dr. Hideharu Numata, Kyoto University, for their guidance throughout this study. I am also thankful to Dr. Toyomi Kotaki, the National Institute of Agrobiological Sciences, for providing technical advice and the insects, Dr. Tetsuro Shinada, Osaka City University, for providing technical advice and $JHSB_3$, Dr. Yoshiaki Tanaka and Dr. Teturo Shinoda, the National Institute of Agrobiological Sciences, for sharing the RNA-seq data of *P. stali*. I would like to thank my colleagues in the Laboratory of Animal Physiology, Osaka City University, for their advice, encouragement and support during this study. I also acknowledge Dr. Masanori Kohda, Osaka City University, for his suggestion during the preparation of this thesis. Part of this work was supported by Grant-in-Aid for JSPS Fellows (DC2, No. 14J06595)

References

Bendena, W.G., Tobe, S.S., 2012. Families of allatoregulator sequences: a 2011 perspective. Canadian Journal of Zoology 90, 521-544.

Bradbury, A.F., Finnie, M.D.A., Smyth, D.G., 1982. Mechanism of C-terminal amide formation by pituitary enzymes. Nature 298, 686-688.

Brown, D.I., Garyfallou, V.T., Urbanski, H.F., 2001. Photoperiodic modulation of GnRH mRNA in the male Syrian hamster. Molecular Brain Research 89, 119-125.

Chiang, A.S., Pszczolkowski, M.A., Lee, C.M., Wei, T.W., 1999. Protocerebral neurons inhibiting proliferation of corpus allatum cells in the cockroach, *Diploptera punctata.* Journal of Comparative Neurology 413, 593-602.

Coast, G.M., Schooley, D.A., 2011. Toward a consensus nomenclature for insect neuropeptides and peptide hormones. Peptides 32, 620-631.

Copenhaver, P.F., Truman, J.W., 1986. Metamorphosis of the cerebral neuroendocrine system in the moth *Manduca sexta*. Journal of Comparative Neurology 249, 186-204.

Denlinger D.L., Yocum G.D., Rinehart J. L., 2005. Hormonal control of diapause. In: Gilbert, L.I., Iatrou, K., Gill, S. (Eds.), Comprehensive Insect Molecular Science, vol 3. Elsevier, pp. 615-650.

Denlinger, D.L., Yocum, G.D., Rinehart, J.P., 2011. Hormonal control of diapause. In: Gilbert, L.I. (Eds.), Insect Endocrinology. Elsevier, pp. 430-463.

Donly, B.C., Ding, Q., Tobe, S.S., Bendena, W.G., 1993. Molecular cloning of the gene for the allatostatin family of neuropeptides from the cockroach *Diploptera punctata*. Proceedings of the National Academy of Sciences of the United States of America 90, 8807-8811.

Girardie, A., 1966. Contrôle de l'activité génitale chez *Locusta migratoria*. Mise en évidence d'un facteur gonadotrope et d'un facteur allatotrope dans la pars intercerebralis. Bulletin de la Société Zoologique de France. 91, 423-439.

Goodman, W.G., Cusson, M., 2012. The juvenile hormones. In: Gilbert, L.I. (Ed.), Insect Endocrinology. Elsevier, pp. 310-365.

Goodman, W.G., Granger, N.A., 2005. The juvenile hormones. In: Gilbert, L.I., Iatrou, K., Gill, S.S. (Eds.), Comprehensive Molecular Insect Science, vol. 3. Elsevier, pp. 319-408.

Granger, N.A., Sturgis, S.L., Ebersohl, R., Geng, C., Sparks T.C., 1996. Dopaminergic control of corpora allata activity in the larval tobacco hornworm, *Manduca sexta*. Archives of Insect Biochemistry and Physiology 32, 449-66.

Hahn, D.A., Denlinger, D.L., 2007. Meeting the energetic demands of insect diapause: Nutrient storage and utilization Journal of Insect Physiology 53, 760-773.

Hodková, M., 1976. Nervous inhibition of corpora allata by photoperiod in *Pyrrhocoris apterus*. Nature 263, 521-523.

Hodková, M., 1979. Hormonal and nervous inhibition of reproduction by brain in diapausing females of *Pyrrhocoris apterus* L. (Hemiptera). Zoologische Jahrbucher. Abteilung fur allgemeine Zoologie und Physiologie der Tiere.

Hodková, M., Okuda, T., Wagner, R., 1996. Stimulation of corpora allata by extract from neuroendocrine complex; comparison of reproducing and diapausing *Pyrrhocoris apterus*. European Journal of Entomology 93, 535-543.

Hummon, A.B., Richmond, T.A., Verleyen, P., Baggerman, G., Huybrechts, J., Ewing, M.A., Vierstraete, E., Rodriguez-Zas, S.L., Schoofs, L., Robinson, G.E., Sweedler, J.V., 2006. From the genome to the proteome: uncovering peptides in the *Apis* brain. Science 314, 647-649.

Huybrechts, J., Bonhomme, J., Minoli, S., Prunier-Leterme, N., Dombrovsky, A., Abdel-Latief, M., Robichon, A., Veenstra, J.A., Tagu, D., 2010. Neuropeptide and neurohormone precursors in the pea aphid, *Acyrthosiphon pisum*. Insect Molecular Biology 19, 87-95.

Ichikawa, T., 1991. Architecture of cerebral neurosecretory cell systems in the silkworm *Bombyx mori*. Journal of Experimental Biology 161, 217-237.

Ikeno, T., Tanaka, S.I., Numata, H., Goto, S.G., 2010. Photoperiodic diapause under the control of circadian clock genes in an insect. BMC Biology 8, 116.

Kaneko, Y., Hiruma, K., 2015. Allatotropin inhibits juvenile hormone biosynthesis by the corpora allata of adult *Bombyx mori*. Journal of Insect Physiology 80, 15-21.

Kang, D.S., Denlinger, D.L., Sim, C., 2014. Suppression of allatotropin simulates reproductive diapause in the mosquito *Culex pipiens*. Journal of Insect Physiology 64, 48-53.

Khan, M.A., Doderer, A., Koopmanschap, A.B., De Kort, C.A.D., 1982. Improved assay conditions for measurement of corpus allatum activity in vitro in the adult Colorado potato beetle, *Leptinotarsa decemlineata*. Journal of Insect Physiology 28, 279-284.

Khan, M.A., Doderer, A., Koopmanschap, A.B., De Kort, C.A.D., 1983. The relative importance of nervous and humoral pathways for control of corpus allatum activity in the adult Colorado potato beetle, *Leptinotarsa decemlineata* (Say). General and Comparative Endocrinology 52, 214-221.

Khan, M.A., Romberg-Privee, H.M., Koopmanschap, A.B., 1986. Location of allatostatic centers in pars lateralis regions of the brain of the Colorado potato beetle. Experientia 42, 836-838.

Kilby, B.A., Youatt, G., 1954. The inhibition of trypsin and chymotrypsin by certain organic phosphorus esters. Biochemical Journal 57, 303-309.

Kogure, M., 1933. The influence of light and temperature on certain characters of the silkworm, *Bombyx mori*. Journal of the Department of Agriculture, Kyushu Imperial University 4, 1-93.

Kotaki, T., 1993. Biosynthetic products by heteropteran corpora allata *in vitro*. Applied Entomology and Zoology 28, 242-245.

Kotaki, T., 1999. Relationships between JH-biosynthetic activity of the corpora allata *in vitro*, their size and adult diapause in a stink bug, *Plautia crossota stali* SCOTT. Entomological Science 2, 307-313.

Kotaki, T., Shinada, T., Kaihara, K., Ohfune, Y., Numata, H., 2009. Structure determination of a new juvenile hormone from a Heteropteran insect. Organic Letter 11, 5234-5237.

Kotaki, T., Shinada, T., Kaihara, K., Ohfune, Y., Numata, H., 2011. Biological activities of juvenile hormone III skipped bisepoxide in last instar nymphs and adults of a stink bug,

Plautia stali. Journal of Insect Physiology 57, 147-152.

Kotaki, T., Yagi, S., 1987. Relationship between diapause development and coloration change in blown-winged green bug, *Plautia stali* Scott (Heteroptera: Pentatomidae). Applied Entomology and Zoology 31, 285-290.

Kotaki, T., Yagi, S., 1989. Hormonal control of adult diapause in the brown-winged green bug, *Plautia stali* SCOTT (Heteroptera: Pentatomidae). Applied Entomology and Zoology 24, 42-51.

Kramer, S.J., Toschi, A., Miller, C.A., Kataoka, H., Quistad, G.B., Li, J.P., Carney, R.L., Schooley, D.A., 1991. Identification of an allatostatin from the tobacco hornworm *Manduca sexta*. Proceedings of the National Academy of Sciences of the United States of America 88, 9458-9462.

Lloyd, G.T., Woodhead, A.P., Stay, B., 2000. Release of neurosecretory granules within the corpus allatum in relation to the regulation of juvenile hormone synthesis in *Diploptera punctata*. Insect Biochemistry and Molecular Biology 30, 739-746.

Lorenz, M.W., Kellner, R., Hoffmann, K.H., 1995. A family of neuropeptides that inhibit juvenile hormone biosynthesis in the cricket, *Gryllus bimaculatus*. Journal of Biological Chemistry 270, 21103-21108.

Marcovitch, S., 1923. Plant lice and light exposure. Science 58, 537-538.

Mizoguchi, A., Ohsumi, S., Kobayashi, K., Okamoto, N., Yamada, N., Tateishi, K., Kataoka, H., 2013. Prothoracicotropic hormone acts as a neuroendocrine switch between pupal diapause

and adult development. PLoS one 8, e60824.

Morita, A., Numata, H., 1999. Location of the photoreceptor for photoperiodism in the stink bug, *Plautia crossota stali*. Physiological Entomology 24, 189-195.

Nation, J.L., 2015. Insect Physiology and Biochemistry, 3rd Edition. CRC press.

Nelson, R.J., Denlinger, D.L., Somers, D.E., 2010. Photoperiodism: The Biological Calendar. Oxford University Press.

Neuhäuser, T., Sorge, D., Stay, B., Hoffmann, K. H., 1994. Responsiveness of the adult cricket (*Gryllus bimaculatus* and *Acheta domesticus*) retrocerebral complex to allatostatin-1 from a cockroach, *Diploptera punctata*. Journal of Comparative Physiology B, 164, 23-31.

Neupert, S., Schattschneider, S., Predel, R., 2009. Allatotropin related peptide in cockroaches: identification via mass spectrometric analysis of single identified neurons. Peptides 30, 489-494.

Nijhout, H.F., 2003. Development and evolution of adaptive polyphenisms. Evolution and Development 5, 9-18.

Numata, H., Shiga, S., Morita, A., 1997. Photoperiodic receptors in arthropods. Zoological Science 14, 187-197.

Okuda, T., Tanaka, S., 1997. An allatostatic factor and juvenile hormone synthesis by corpora allata in *Locusta migratoria*. Journal of Insect Physiology 43, 635-641.

Ons, S., Richter, F., Urlaub, H., Pomar, R.R., 2009. The neuropeptidome of *Rhodnius prolixus* brain. Proteomics 9, 788-792.

Ons, S., Sterkel, M., Diambra, L., Urlaub, H., Pomar, R.R. 2011. Neuropeptide precursor gene discovery in the Chagas disease vector *Rhodnius prolixus*. Insect Molecular Biology 20, 29-44.

Pavelka, J., Shimada, K., Kostal, V., 2003. TIMELESS: a link between fly's circadian and photoperiodic clocks?. European Journal of Entomology 100, 255-266.

Perera, A.D., Follett, B.K., 1992. Photoperiodic induction in vitro: the dynamics of gonadotropin-releasing hormone release from hypothalamic explants of the Japanese quail. Endocrinology 131, 2898-2908.

Poras, M., 1982. Le contrôle endcrinien de la diapause imaginale des femelles de *Tetrix undulata* (Sowerby, 1806) (Orthoptere, Tetrigidae). General and Comparative Endocrinology 46, 200-210.

Poras, M., Beahr, J.C., Cassier, P., 1983. Control of corpus allatum activity during imaginal diapause in females of *Locusta migratoria*. International Journal of Invertebrate Reproduction 6, 111-122.

Pratt, G.E., Farnsworth, D.E., Feyereisen, R., 1990. Changes in the sensitivity of adult cockroach corpora allata to a brain allatostatin. Molecular and cellular endocrinology 70, 185-195.

Pratt, G.E., Farnsworth, D.E., Fok, K.F., Siegel, N.R., McCormack, A.L., Shabanowitz, J., Hunt, D.F., Feyereisen, R., 1991. Identity of a second type of allatostatin from cockroach brains: an octadecapeptide amide with a tyrosine-rich address sequence. Proceedings of the National

Academy of Sciences of the United States of America 88, 2412-2416.

Pratt, G.E., Tobe, S.S., 1974. Juvenile hormones radiobiosynthesised by corpora allata of adult female locusts *in vitro*. Life Sciences 14, 575-586.

Raabe, M., 1989. Recent developments in insect neurohormones. Plenum Press.

Rachinsky, A., 1994. Octopamine and serotonin influence on corpora allata activity in honey bee (*Apis mellifera*) larvae. Journal of Insect Physiology 40, 549-554.

Raikhel, A.S., Brown, M.R., Belles, X., 2005. Hormonal control of reproductive processes. In: Gilbert, L.I., Iatrou, K., Gill, S. (Eds.), Comprehensive Molecular Insect Science, vol 3. Elsevier, pp. 433-491.

Readio, J., Chen, M.H., Meola, R., 1999. Juvenile Hormone Biosynthesis in Diapausing and Nondiapausing *Culex pipiens* (Diptera: Culicidae). Journal of medical entomology 36, 355-360.

Röller, H., Dahm, K.H., Sweely, C.C., Trost, B.M., 1967. The structure of the juvenile hormone. Angewandte Chemie International Edition in English 6, 179-180.

Sakamoto, T., Uryu, O., Tomioka, K., 2009. The clock gene *period* plays an essential role in photoperiodic control of nymphal development in the cricket *Modicogryllus siamensis*. Journal of Biological Rhythms 24, 379-390.

Sanders, D.S., 2002. Insect Clocks, 3rd Edition. Elsevier.

Shiga, S., Numata, H., 2000. The role of neurosecretory neurons in the pars intercerebralis and pars lateralis in reproductive diapause of the blowfly, *Protophormia terraenovae*.

Naturwissenschaften 87, 125-128.

Shiga, S., Toyoda, I., Numata, H., 2000. Neurons projecting to the retrocerebral complex of the adult blow fly, *Protophormia terraenovae.* Cell and Tissue Research 299, 427-439.

Shiga, S., Hamanaka, Y., Tatsu, Y., Okuda, T., Numata, H., 2003. Juvenile hormone biosynthesis in diapause and nondiapause females of the adult blow fly *Protophormia terraenovae*. Zoological Science 20, 1199-1206.

Shimokawa, K., Numata, H., Shiga, S., 2008. Neurons important for the photoperiodic control of diapause in the bean bug, *Riptortus pedestris*. Journal of Comparative Physiology A 194, 751-762.

Stay, B., Chan, K.K., Woodhead, P., 1992. Allatostatin-immunoreactive neurons projecting to the corpora allata of adult *Diploptera punctata*. Cell and Tissue Research 270, 15-23.

Stay, B., Tobe, S.S, Bendena, W.G., 1995 Allatostatins: identification, primary structures, functions and distribution. Advances in Insect Physiology 25, 267-337.

Thompson, C.S., Yagi, K.J., Chen, Z.F., Tobe, S.S., 1990. The effects of octopamine on juvenile hormone biosynthesis, electrophysiology, and cAMP content of the corpora allata of the cockroach *Diploptera punctata.* Journal of Comparative Physiology B 160, 241-249.

Tobe, S.S., 1980. Regulation of the corpora allata in adult female insects. In: Locke M., Smith D.S. (Eds.) Insect Biology in the Future. Academic Press, pp. 345-368.

Tobe, S.S., Pratt, G.E., 1974. The influence of substrate concentrations on the rate of insect juvenile hormone biosynthesis by corpora allata of the desert locust *in vitro*. Biochemical

Journal 144, 107-113.

Virant-Doberlet, M., Horseman, G., Loher, W., Huber, F., 1994. Neurons projecting from the brain to the corpora allata in orthopteroid insects: anatomy and physiology. Cell and Tissue Research 277, 39-50.

Wang, J., Meyering-Vos, M., Hoffmann, K.H., 2004. Cloning and tissue-specific localization of cricket-type allatostatins from *Gryllus bimaculatus*. Molecular and Cellular Endocrinology 227, 41-51.

Wigglesworth, V.B., 1970. Insect Hormones. W. H. Freeman and Company.

Williams, C.M., 1961. The juvenile hormone. II. Its role in the endocrine control of molting, pupation, and adult development in the Cecropia silkworm. The Biological Bulletin 121, 572-585.

Witek, G., Verhaert, P., Lorenz, M.W., Hoffmann, K.H., 1999. Immunolocalization of two types of allatostatins in the central nervous system of the cricket *Gryllus bimaculatus* (Ensifera: Gryllidae). European Journal of Entomology 96, 279-286.

Woodhead, A.P., Asano, W.Y., Stay, B., 1993. Allatostatins in the haemolymph of *Diploptera punctata* and their effect in vivo. Journal of Insect Physiology 39, 1001-1005.

Woodhead, A.P., Stay, B., Seidel, S.L., Khan, M.A., Tobe, S.S., 1989. Primary structure of four allatostatins: neuropeptide inhibitors of juvenile hormone synthesis. Proceedings of the National Academy of Sciences of the United States of America 86, 5997-6001.

Yamamura, T., Hirunagi, K., Ebihara, S., Yoshimura, T., 2004. Seasonal morphological

changes in the neuro-glial interaction between gonadotropin-releasing hormone nerve terminals and glial endfeet in Japanese quail. Endocrinology 145, 4264-4267.

Yamanaka, N., Yamamoto, S., Žitňan, D., Watanabe, K., Kawada, T., Satake, H., Kaneko, Y., Hiruma, K., Tanaka, Y., Shinoda, T., Kataoka, H., 2008. Neuropeptide receptor transcriptome reveals unidentified neuroendocrine pathways. PLoS ONE 3: e3048.

Yellon, S.M., 1994. Effects of photoperiod on reproduction and the gonadotropin-releasing hormone-immunoreactive neuron system in the postpubertal male Djungarian hamster. Biology of Reproduction 50, 368-372.

Table 1-1. Number of somata in the brain backfilled from the corpus cardiacum-corpus allatum complex in *Plautia stali*. The numbers shown for the left and right hemispheres are number of somata per individual (*a*-*o*, *N* = 15). A drawing of the neuron in *k* is shown in Figure 1-6A.

	Left				Right			
	PL-d	PL-v	PI	NCC2	NCC2	PI	PL-v	PL-d
a	2	5	8	3	1	5	5	3
b	2	4	0	4	2	7	0	2
c	0	2	9	5	0	8	0	2
d	2	3	6	3	1	7	1	0
e	3	6	7	4	5	8	6	3
f	2	3	4	5	4	1	2	0
g	2	2	18	5	0	14	0	0
h	0	4	6	4	0	9	0	2
i	3	4	13	4	0	12	0	0
j	0	3	7	3	0	15	0	0
k	2	3	12	5	4	12	3	3
l	0	2	11	2	3	8	2	0
m	1	2	6	3	0	13	0	0
n	2	2	9	4	5	9	6	1
o	1	4	0	1	2	0	7	1
Max	3	6	18	5	5	15	7	3

Table 2-1. Peptides and monoamines used for radiochemical assay of JH biosynthesis.

		Amino acid sequences [a]	Incubation 3 times [b]	Incubation once [c]	*N*	Concentrations (M)	References
Peptides [d]	Dippu-AST2	A Y S Y V S E Y K R L P V Y N F G L -NH_2	○		1-2	10^{-8}, 10^{-6}, 10^{-4}	Pratt et al., 1990
	Dippu-AST8	G G S L Y S F G L -NH_2	○	○	5-6	10^{-9}, 10^{-8}, 10^{-7}, 10^{-5}	Woodhead et al., 1989
	Dippu-AST9	G D G R L Y A F G L -NH_2		○	5-6	2.5×10^{-5}, 2.5×10^{-9}	Woodhead et al., 1989
	Grybi-MIP/AST1	A W R D L S G G W -NH_2	○		10	10^{-8}, 10^{-6}	Lorenz et al., 1995
	Plast-MIP/AST1	A W K D L S K A W -NH_2	○		9-10	10^{-8}, 10^{-6}, 10^{-4}	Current paper
	Apime-AST	S Y W K Q C A F N A V S C F -NH_2	○		3-4	10^{-8}, 10^{-6}, 10^{-4}	Hummon et al., 2006
	Peram-AT	G F K N V A L S T A R G F -NH_2	○		4	10^{-8}, 10^{-6}, 10^{-4}	Neupert et al., 2009
	Plast-sNPF	N S N R S P Q L R L R F -NH_2	○		3-4	10^{-8}, 10^{-6}, 10^{-4}	Current paper
Amines	octopamine	—	○		11-12	10^{-8}, 10^{-6}	Thompson et al., 1990
	dopamine	—	○		3-5	10^{-10}, 10^{-8}, 10^{-6}, 10^{-4}	Granger et al., 1996
	serotonin	—	○		3-5	10^{-10}, 10^{-8}, 10^{-6}, 10^{-4}	Rachinsky, 1994

[a] Amino acids are shown by single letter codes.

[b] The CC-CA was incubated 3 times for 3 h. In the 2nd incubation, a peptide or an amine was added in the medium.

[c] The CC-CA was incubation once for 3 h with a peptide.

[d] I adopt nomenclature of peptides suggested by Coast and Schooley (2011).

Table 2-2. Sequences of primers.

Oligonucletides		Sequence (5' >> 3')
Plast-Mip/Ast	Ast-F	AAT CCC AGG AGG ATC ACA TTT C
	Ast-R	GGT CTT TCC ACG CTC TCT TT
RpL32	RpL32-F	GAA TTG GCG CAA GCC TAA AG
	RpL32-R	TTG CGG AAA CCA GTA GGA AG

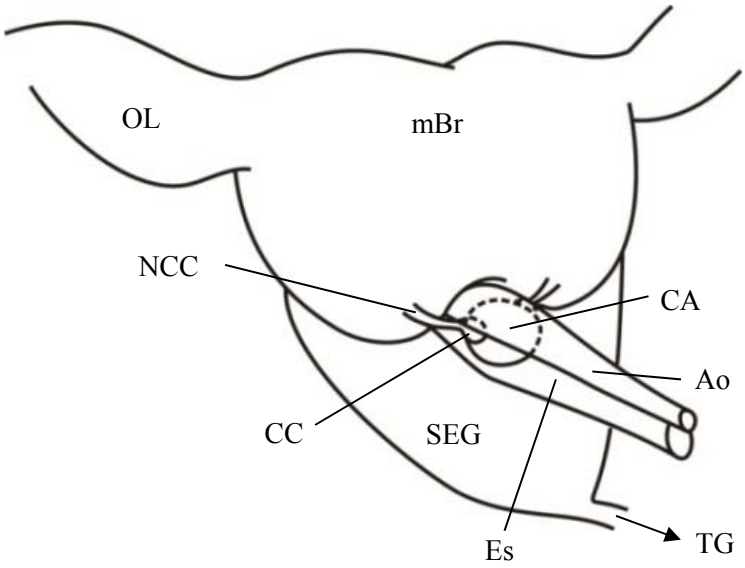

Fig. 1-1. Schematic illustration of the cephalic ganglion and retrocerebral complex of *Plautia stali* (posterior view). The brain is composed of a middle part (mBr) and optic lobes (OL). A complex of the corpus allatum (CA) and bilateral corpora cardiaca (CC) is located between the dorsal aorta (Ao) and the ventral esophagus (Es). The complex of the CC and CA is connected to the brain via the nervi corporis cardiaci (NCC). The cervical connective from the subesophageal ganglion (SEG) extends to the thoracic ganglia (TG).

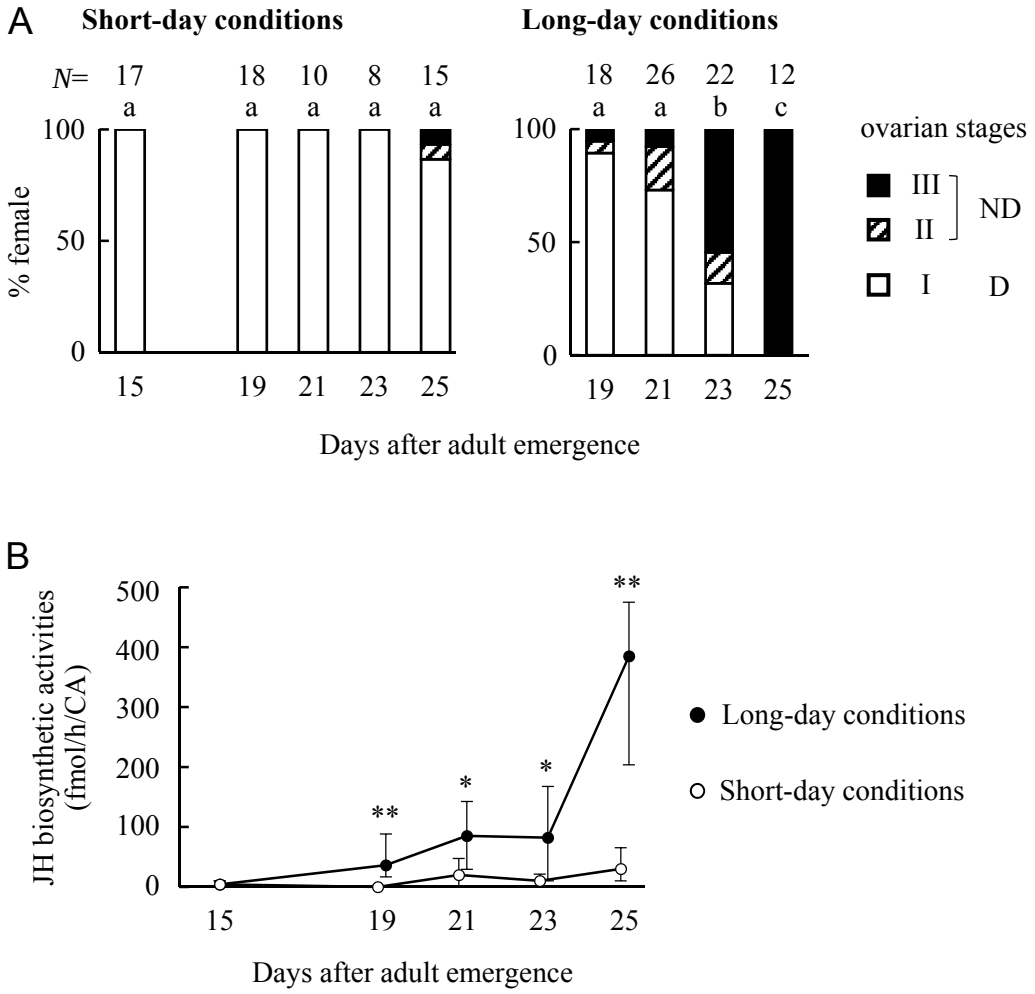

Fig. 1-2. Ovarian development (A) and JH biosynthetic activities (B) under short-day and long-day conditions in adult females of *Plautia stali*. Females were reared under short-day conditions until day 15 and then transferred to long-day conditions or retained under short-day conditions. (A) Under short-day conditions, most females had immature ovaries; under long-day conditions, the ovaries gradually developed. Diapause females (D) had ovaries at stage I, and non-diapause females (ND) had ovaries at stage II or III. Columns with different letters show significant differences in the three ovarian stages among the nine groups under the two photoperiod conditions (Steel-Dwass test, $P < 0.05$). (B) Medians and interquartile ranges of JH biosynthetic activities in females are shown. The activities remained low under short-day conditions, while they increased under long-day conditions. On days 19, 21, 23, and 25, the activities under long-day conditions were significantly higher than those under short-day conditions (Mann-Whitney U test, $*P < 0.05$, $**P < 0.01$). N = 8-26.

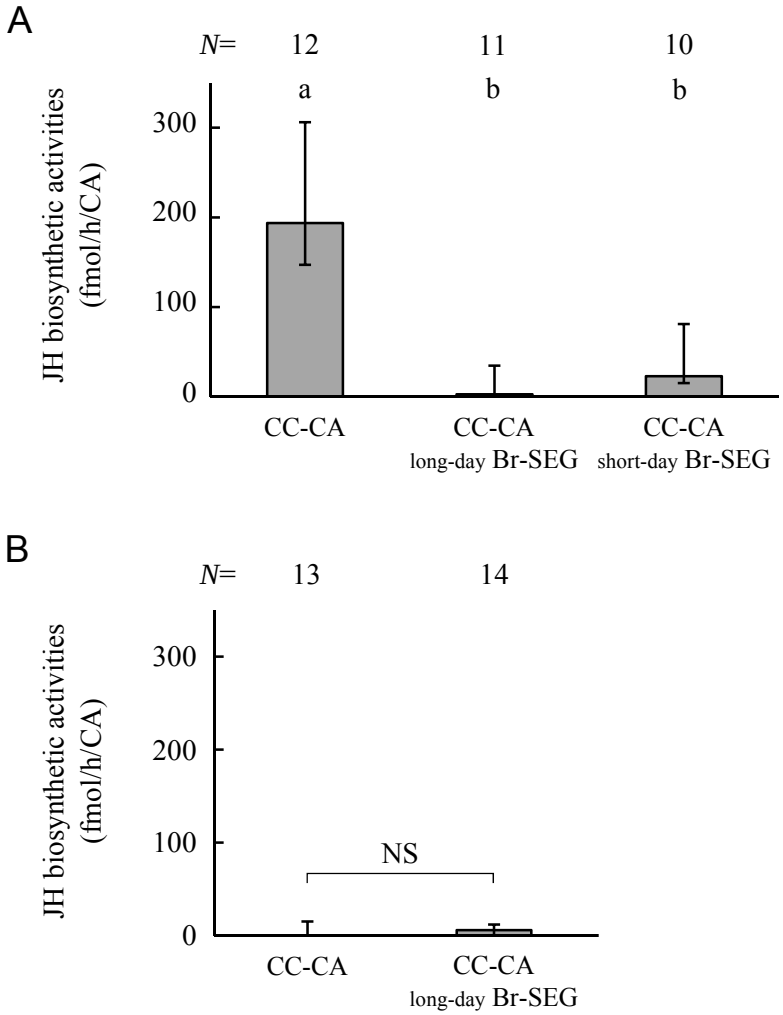

Fig. 1-3. Effects of the brain and subesophageal complex (Br-SEG) on JH biosynthetic activities in adult females of *Plautia stali*. Medians and interquartile ranges of JH biosynthetic activities are shown. (A) Effects of the Br-SEG on the JH biosynthetic activities of the corpus allatum (CA) from long-day females. The corpus cardiacum (CC)-corpus allatum complex was coincubated with the Br-SEG from long-day or short-day females. Both long-day and short-day Br-SEG showed inhibitory effects (Steel-Dwass test, $P < 0.05$). (B) Effects of the Br-SEG from long-day females on the JH biosynthetic activities of the CA from short-day females. The CC-CA was coincubated with the Br-SEG from long-day females. No significant effects (NS) were observed. (Mann-Whitney U test, $P < 0.05$).

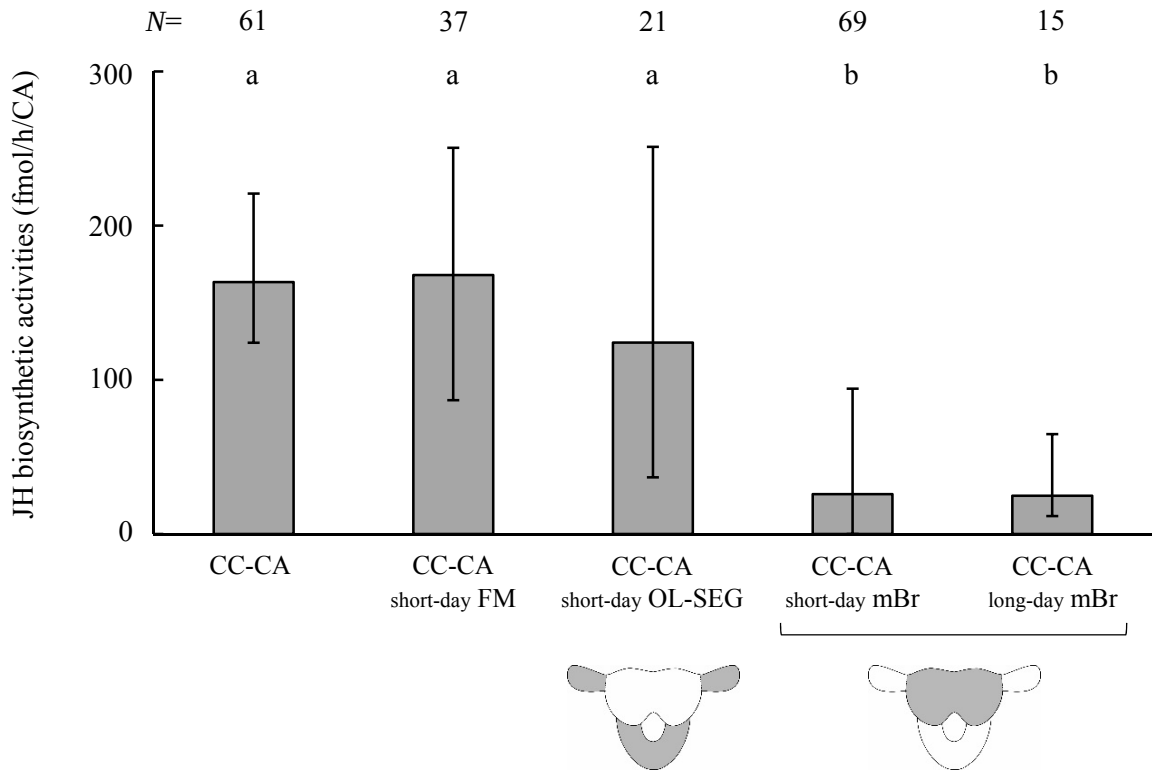

Fig. 1-4. Effects of different parts of the cephalic ganglia on the JH biosynthetic activities of the corpus allatum (CA) from long-day females. Medians and interquartile ranges of JH biosynthetic activities are shown. The CC-CA was coincubated with the flight muscles (FM), the optic lobe with subesophageal ganglion (OL-SEG), or the middle part of the brain (mBr). Neither the FM nor the OL-SEG from short-day females showed significant effects. The mBr from short-day and long-day females exerted inhibitory effects. Columns with different letters show significant differences in JH biosynthetic activities among the five groups (Steel-Dwass test, $P < 0.05$).

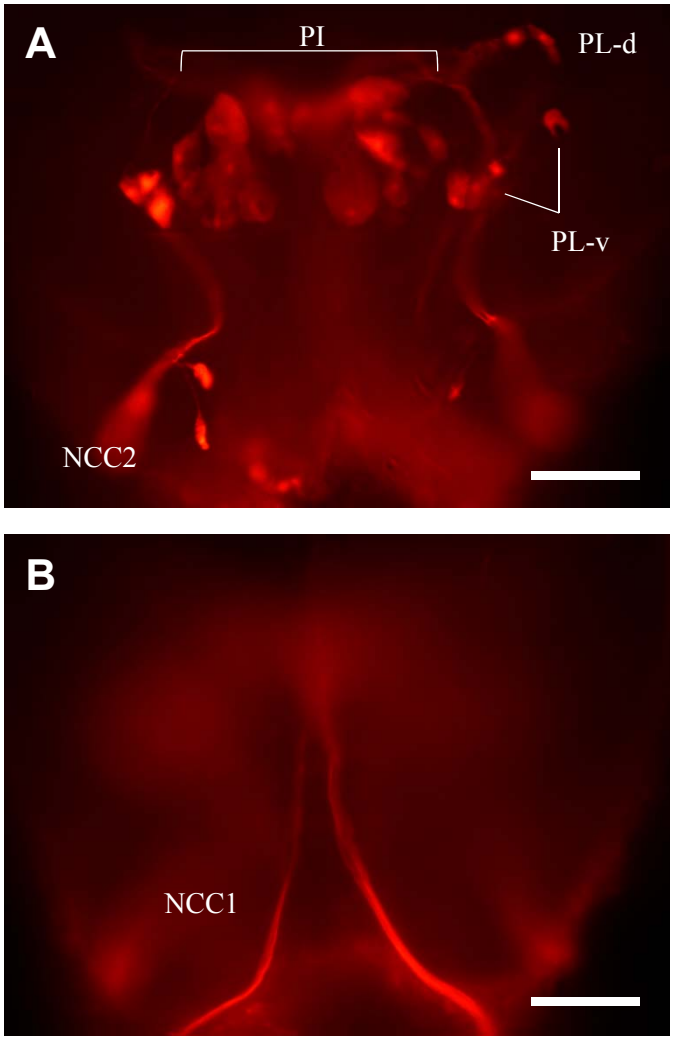

Fig. 1-5. Posterior view of brain neurons backfilled from the corpus cardiacum-corpus allatum complex of *Plautia stali* (in whole mount, upper is to dorsal). All neurons were not stained. (A) A montage of three photos from different depths. Somata in the pars intercerebralis (PI), in the pars lateralis (PL), and on the nervi corporis cardiac 2 (NCC2) were stained. Two clusters of somata, PL-d and PL-v, were revealed in the PL. (B) The NCC1 from the somata in the PI of the same brain as in (A) are in focus at an anterior region. Scales = 100 μm.

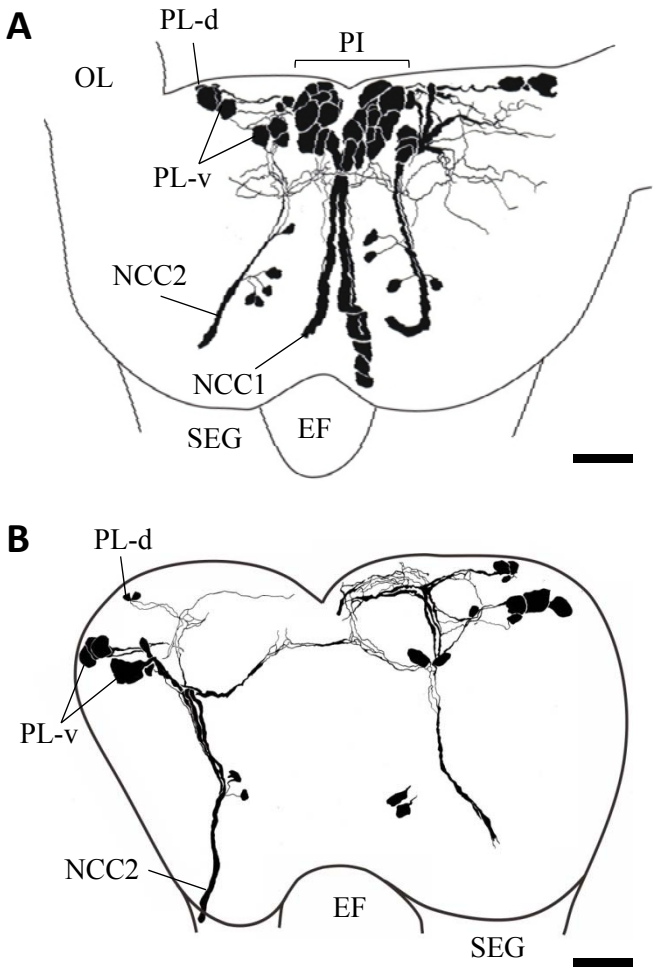

Fig. 1-6. Drawings of brain neurons bilaterally backfilled from the corpus cardiacum-corpus allatum complex of *Plautia stali*. Dorsal is to top. (A) Neurons were bilaterally backfilled. In the pars lateralis (PL), somata of PL-d neurons are located anterior and dorsal to those of PL-v neurons. Pars intercerebralis (PI) and PL neurons projected to the corpus allatum-corpus cardiacum complex through the nervi corporis cardiac 1 (NCC1) and NCC2, respectively. (B) Neurons were bilaterally backfilled after severance of the NCC1. Only PL-d and PL-v neurons and those with somata on the NCC2 were stained. Fibers from the PL neurons intersect at a posterior region of the PI. Arrow heads indicate the somata OL, optic lobe; EF, esophageal foramen; SEG, subesophageal ganglion. Scales = 100 μm.

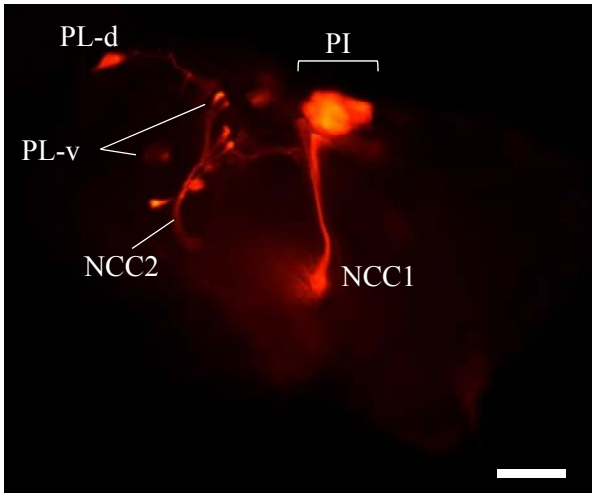

Fig. 1-7. Photomicrograph of a posterior view of brain neurons unilaterally backfilled from the corpus cardiacum-corpus allatum complex of *Plautia stali* (whole mount). A montage of an anterior plane including NCC1 and three posterior planes including somata. The nervi corporis cardiac 1(NCC1) and NCC2 were stained only in the left hemisphere. Somata in the pars lateralis (PL), designated as PL-d and PL-v neurons, and those on the NCC2 were ipsilaterally stained. Somata of the pars intercerebralis (PI) neurons were contralaterally stained. Scale = 100 μm.

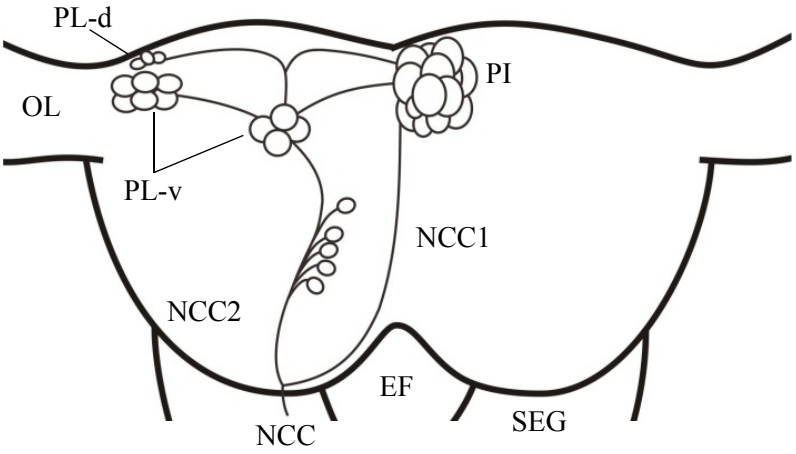

Fig. 1-8. Schematic illustration of neurons in the pars intercerebralis (PI), in the pars lateralis (PL), and on the nervi corporis cardiac 2 (NCC2) projecting to the corpus cardiacum-corpus allatum complex of *Plautia stali*. Only the neurons exiting to the left NCC are shown. PI and PL neurons project to the corpus cardiacum-corpus allatum complex through the contralateral NCC1 and the ipsilateral NCC2, respectively. OL, optic lobe; EF, esophageal foramen; SEG, subesophageal ganglion.

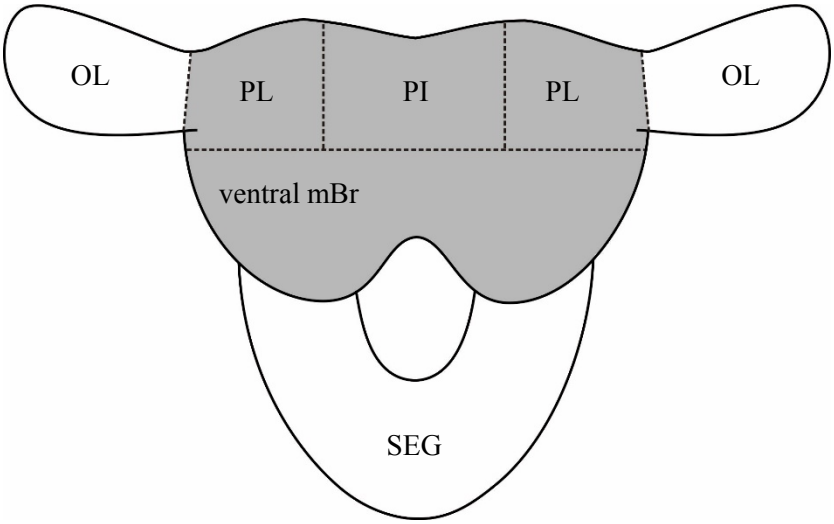

Fig. 2-1. Schematic illustration of regions of the cephalic ganglia used for tissue extraction in *Plautia stali*. The cephalic ganglia is divided into a middle part of the brain (mBr, gray), optic lobes (OL) and subesophageal ganglion (SEG). The mBr is further separated to the pars intercerebralis (PI), the pars lateralis (PL), and a ventral region of mBr (ventral mBr).

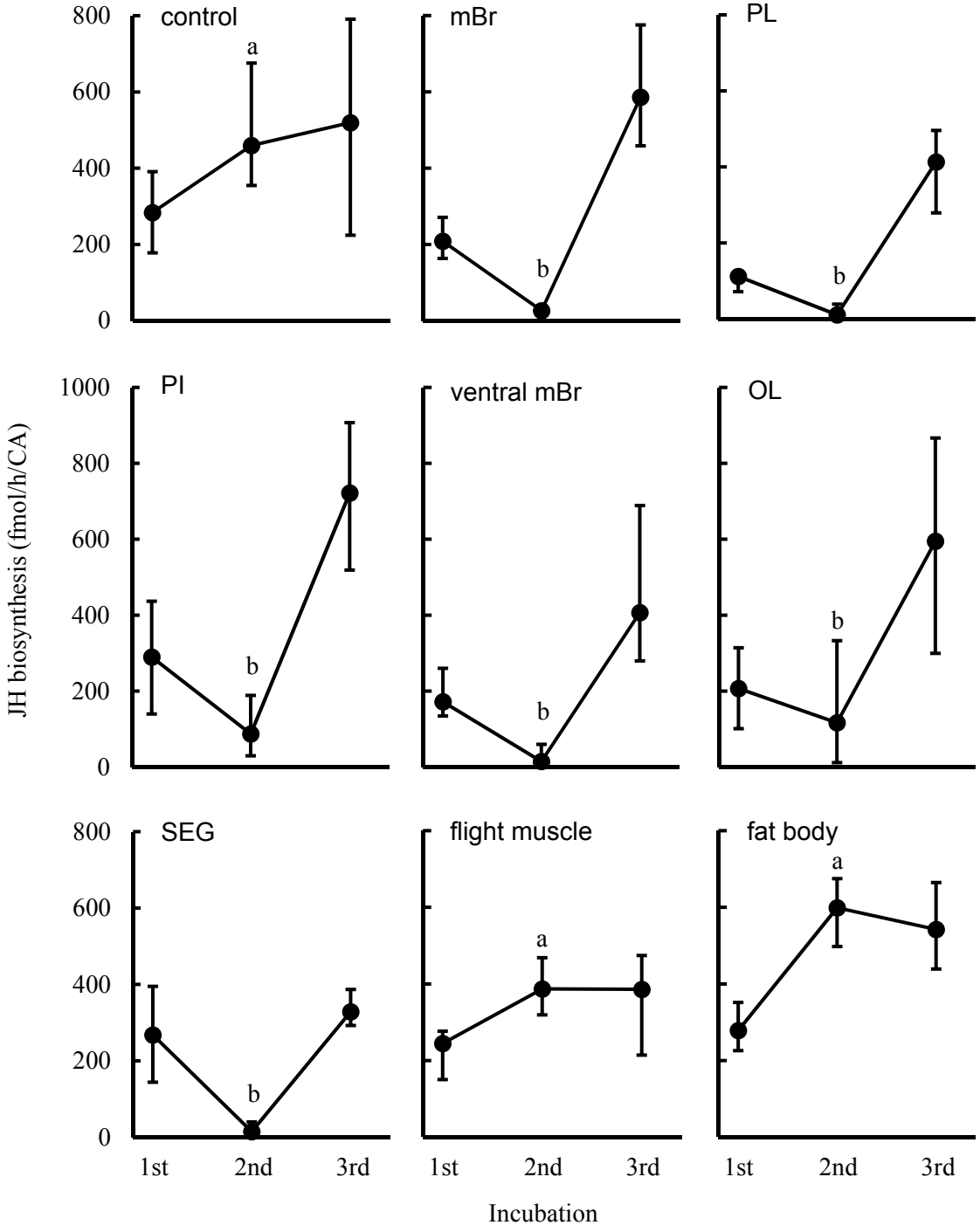

Fig. 2-2. Effects of extracts of the cephalic ganglia on JH biosynthetic activities in *Plautia stali*. Medians and interquartile ranges of JH biosynthetic activities are shown. In control, the corpus allatum and corpus cardiacum complex was incubated three times in the medium without the extracts. In test groups, extract of the middle part of the brain (mBr), pars lateralis (PL), pars intererecerebralis (PI), a ventral region of the mBr, optic lobes (OL), subesophageal gangion (SEG), flight muscles or fat body was added to the second incubation medium. JH biosynthetic activities at the second incubation with different letters show significant difference in the percentage inhibition (see text) among the nine groups (Steel-Dwass test, $P < 0.05$). Medians and interquartile ranges of JH biosynthetic activities are shown. $N = 4$-17.

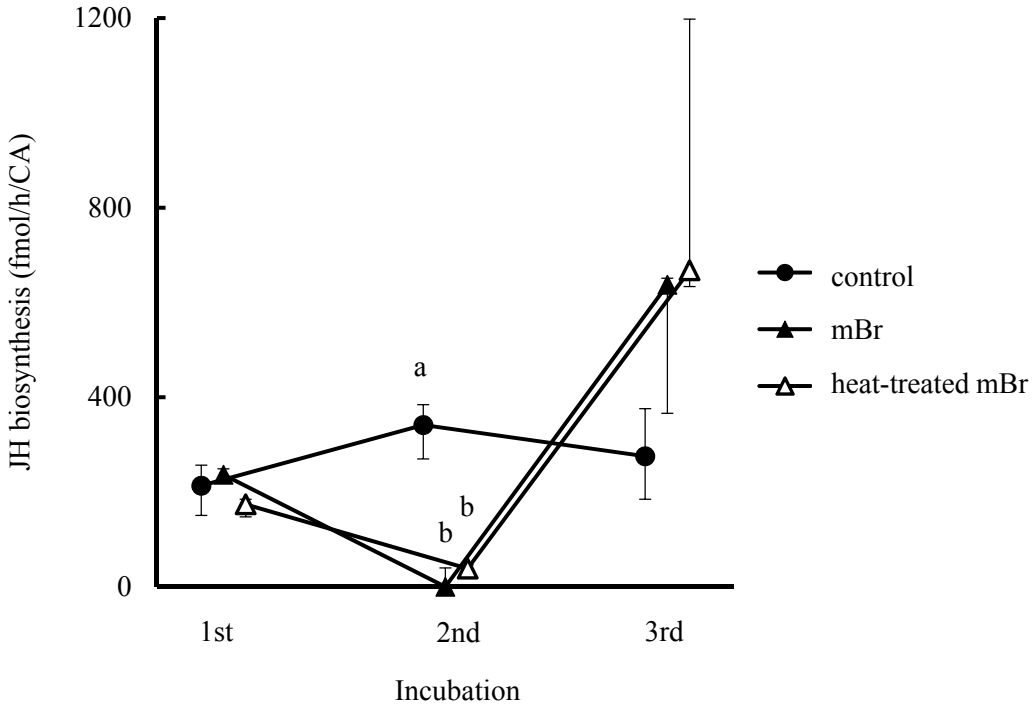

Fig. 2-3. Effects of heat-treatment on the allatostatic activity of the extracts of the middle part of the brain (mBr) in *Plautia stali*. Medians and interquartile ranges of JH biosynthetic activities are shown. The corpus cadiacum and corpus allatum complex was incubated three times to examine JH biosynthetic activities. In the second incubation the extracts of the mBr without heat-treatment or those with heat-treatment was added. JH biosynthetic activities at the second incubation with different letters show significant difference in the percentage inhibition (see text) among three groups (Steel-Dwass test, $P < 0.05$). Medians and interquartile ranges of JH biosynthetic activities are shown. $N = 4$-5.

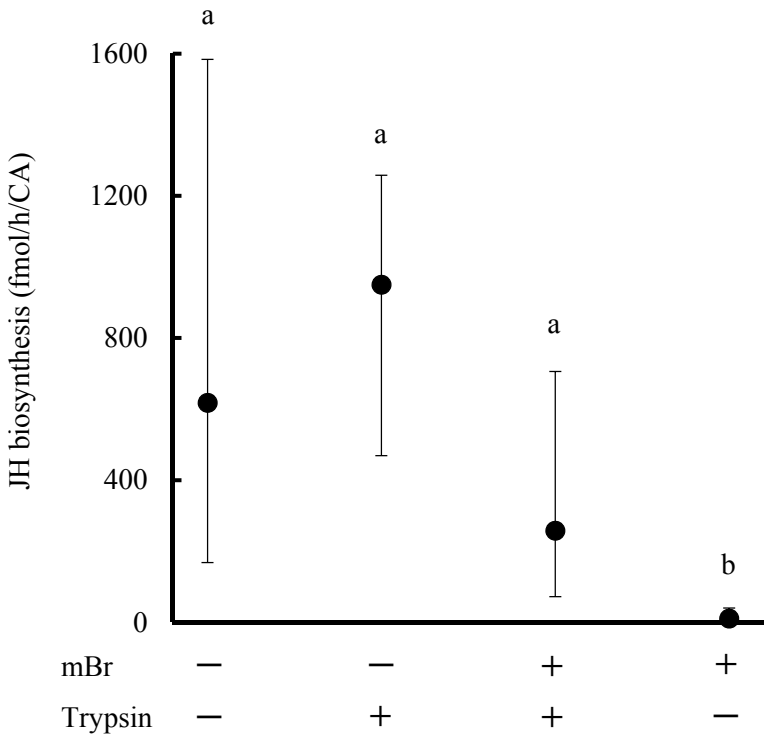

Fig. 2-4. Effects of trypsin-treatment on the allatostatic activity of the extracts of the middle part of the brain (mBr) in *Plautia stali*. Medians and interquartile ranges of JH biosynthetic activities are shown. The corpus cadiacum and corpus allatum complex was incubated in medium without extracts and trypsin, medium with inactivated trypsin, medium with trypsin-treated extracts, and medium with intact extracts. JH biosynthetic activities with different letters show a significant difference in the percentage inhibition (see text) among four groups (Steel-Dwass test, $P < 0.05$). Medians and interquartile ranges of JH biosynthetic activities are shown. $N = 13$-14.

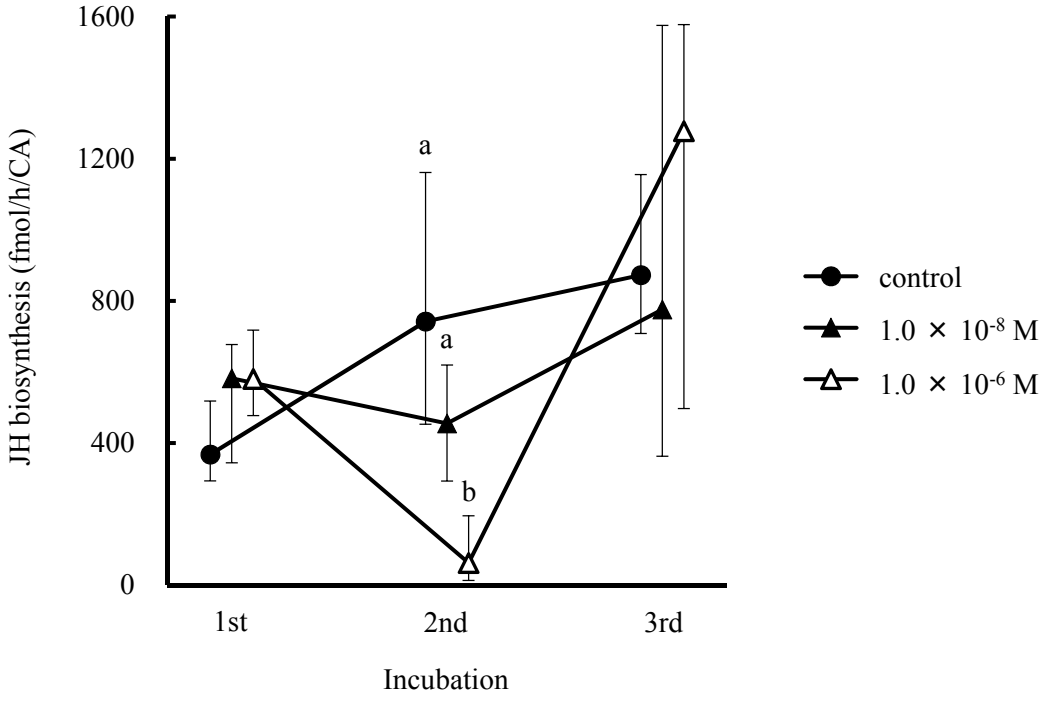

Fig. 2-5. Effects of Grybi-MIP/AST1 on JH biosynthetic activities in *Plautia stali*. Medians and interquartile ranges of JH biosynthetic activities are shown. The corpus cadiacum and corpus allatum complex was incubated three times to examine JH biosynthetic activities. In the second incubation, Grybi-MIP/AST1 was added. JH biosynthetic activities at the second incubation with different letters show significantly difference in the percentage inhibition (see text) among three groups (Steel-Dwass test, $P < 0.05$). Medians and interquartile ranges of JH biosynthetic activities are shown. $N = 10$.

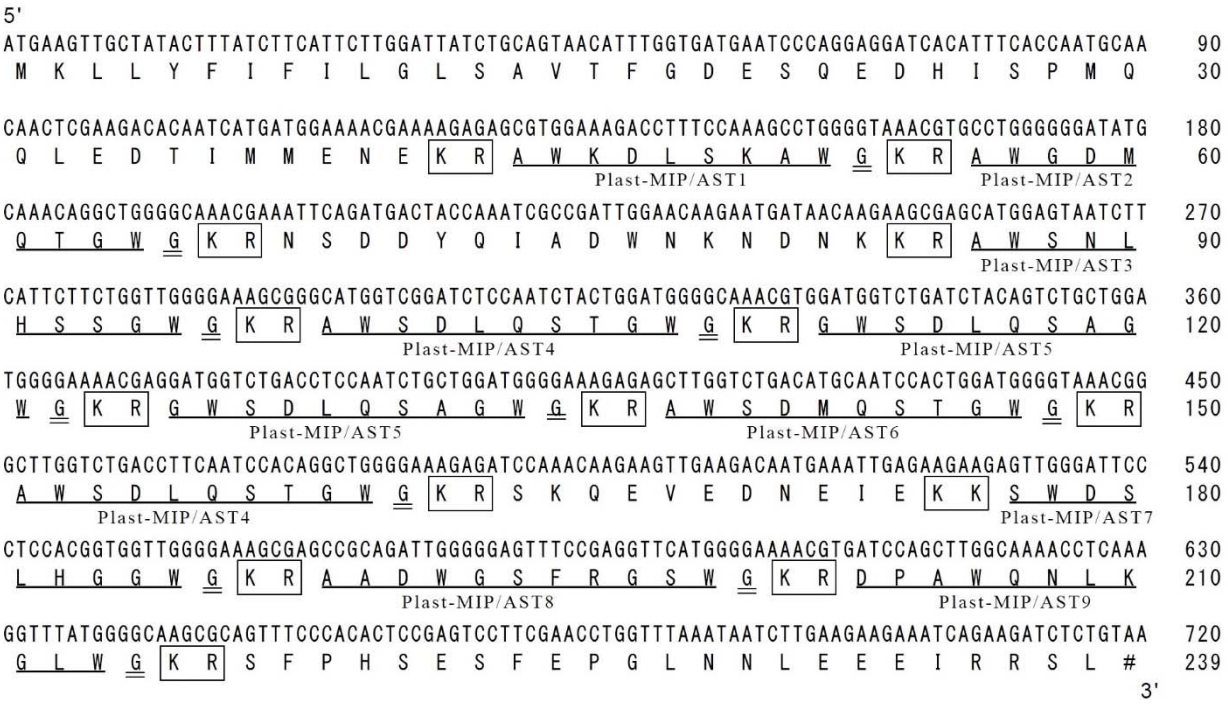

Fig. 2-6. Sequences of the nucleotide of the open reading frame and deduced amino acids of a B-type allatostatin (MIP/AST) precursor, named Plast-MIP/AST, in *Plautia stali*. Numbers of nucleotides and amino acids are shown on the right. Eleven peptide sequences underlined are proposed as allatostatins. Potential cleavage sites are boxed and glycine residues required for amidation are double underlined. # represents stop codon.

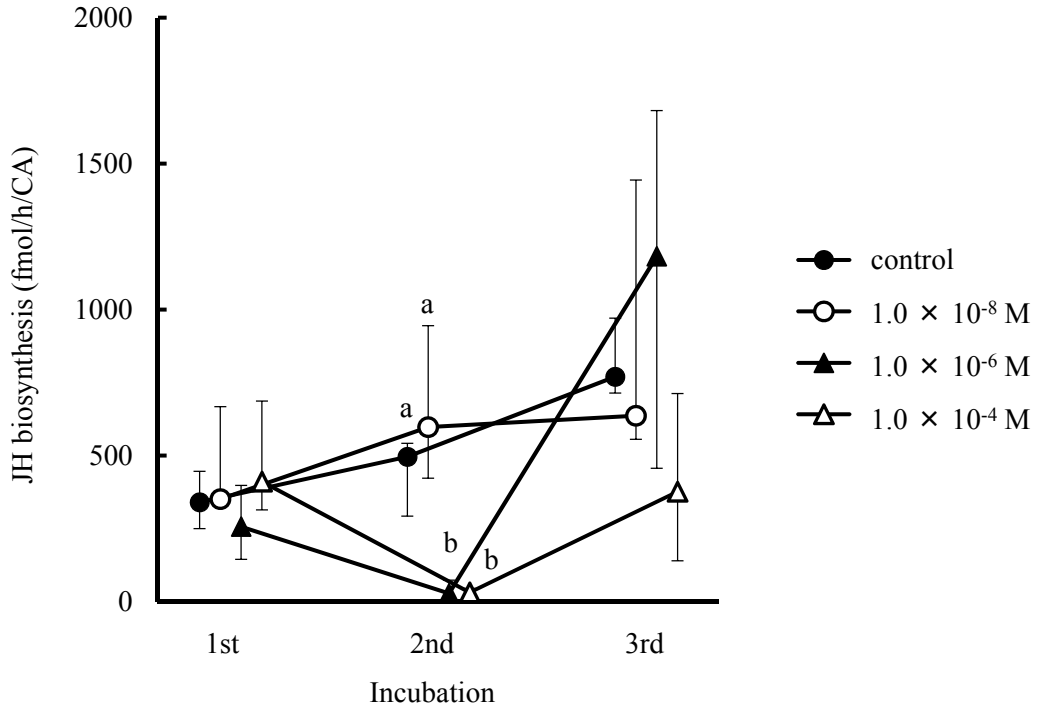

Fig. 2-7. Effects of Plast-MIP/AST1 on JH biosynthetic activities in *Plautia stali*. Medians and interquartile ranges of JH biosynthetic activities are shown. The corpus cadiacum and corpus allatum complex was incubated three times to examine JH biosynthetic activities. In the second incubation, Plast-MIP/AST1 was added. JH biosynthetic activities at the second incubation with different letters show significant difference in the percentage inhibition (see text) among four groups (Steel-Dwass test, $P < 0.05$). Medians and interquartile ranges of JH biosynthetic activities are shown. $N = 9$-10.

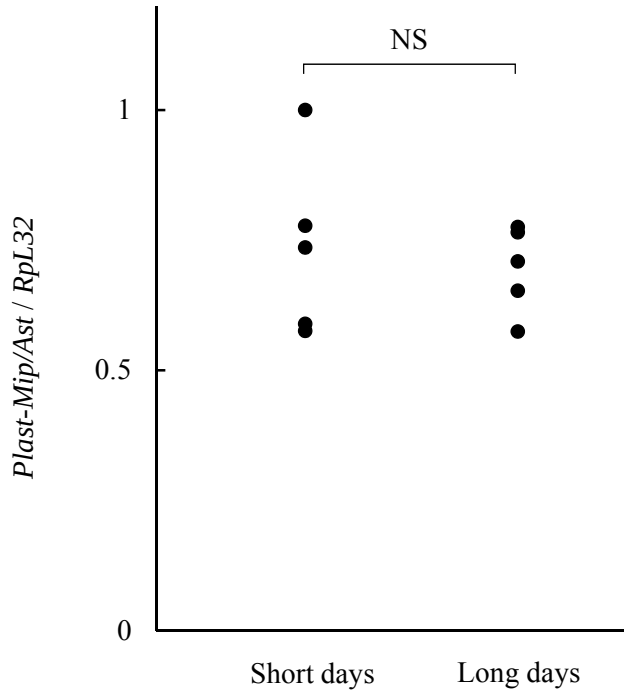

Fig. 2-8. Expression levels of *Plast-Mip/Ast* mRNA in the middle part of the brain (mBr in figure 1-1) derived from short-day and long-day conditions in *Plautia stali*. The mRNA levels of *Plast-Mip/Ast* were assessed by quantitative real-time PCR and normalized to *RpL32*. There is no significant difference between two groups (Mann- Whitney U test, $P > 0.05$). $N = 5$.