Chapter 6   Relationships between the JH-biosynthetic activity and

volume of the corpus allatum

The juvenile hormone (JH) serves as a gonadotropin, and a deficiency or reduced

haemolymph titre of JH is responsible for the occurrence of adult diapause in many

species (Denlinger, 1985; Wyatt and Davey, 1996). Chapter 3 suggests that a similar

mechanism appears to control adult diapause in *P. c. stali*. Measuring JH titres in this

species is necessary to test this hypothesis. However, as indicated in Chapters 4 and 5,

putative JH in this bug seems different from any known JHs. For this reason, thus far,

no way is available to determine JH titres. Chapters 4 and 5, instead, suggest that the *in*

vitro incubation technique used in these chapters can be adopted to measure the

JH-biosynthetic activity of CA, even if the chemical structure of the JH is unknown.

One of the main purposes of this chapter is to determine the JH-biosynthetic activity

using this technique (RCA) in relation to the presence or absence of diapause in *P. c.*

stali.

The size of CA is often used as an indicator of JH-biosynthetic activity in the

adult stage though this does not always hold true (Cassier, 1979, 1990; Tobe and Stay,

1985). In *P. c. stali*, large CA are associated with reproductively active adults and small

CA with diapause ones. However, diapause adults of this bug develop ovaries and

ectodermal accessory glands in females and males, respectively, without increasing the

CA size if their nervous connections between the brain and CA are severed (Chapter 3).

In this chapter, the CA size and activity were measured for nerve-transected females to

examine how such an operation would influence the relationship between the two

variables.

Reproductively active adults of *P. c. stali* are green in body colour but their body colour turns brown if they enter diapause. This colour change is reversible: brown bugs turn green slowly as diapause ends, as shown in Chapters 1 and 2. Because allatectomy causes green bugs to turn brown (Chapter 3), the CA appear to be responsible for the control of body colour in this bug. In this chapter, the CA activity was compared among females with various body colours to establish the role of JH in the control of body colour.

Materials and Methods

A stock culture of *P. c. stali* was established from adults collected in a mulberry field in Tsukuba, Ibaraki in 1996. Bugs were reared as already described. The experimental method was the same as the one in Chapter 3-5, except that the shape of CA was assumed to be ellipsoid, and the following formula was used for estimating the CA volume:

$V=4/3 \times D_1 \times D_2 \times (D_1+D_2)/2 \times (1/8) \times 3.14,$

where D_1 and D_2 were the diameters along and across the body axis, respectively, and the remaining diameter was assumed to be an average of the other two diameters. To determine radioactivity, either liquid scintillation counter (Beckman) or radio-TLC analyser (Raytest) was used. The oocyte size was represented by the maximal diameter of ovarioles containing the terminal oocyte. To select a suitable incubation medium, CA from reproductively active females were cultured in three different media, MEM, TC 199, and Grace's medium. Three successive 2-h incubations of CA were conducted to

test for the linearity of JH biosynthesis.

Results

Conditions for CA incubation

Among the three incubation media used, MEM was the best medium for CA incubation in terms of the production of the radiolabelled JH-active substance or JH with an Rf value of 0.5. During a 2-h incubation, the CA in MEM released an average amount of 6,625 dpm (SD=1,951 dpm, n=5) of radiolabelled products, whereas those in TC199 and Grace's released an average of 2,332±824 dpm and 312±79 dpm, respectively. Time course analysis where CA were subjected to three successive 2-h incubations showed that the CA cultured in MEM produced more JH during a second 2-h incubation period (2-4 h) than during the first 2-h period ($p<0.05$, Tukey HSD test; Fig. 25). During the last 2-h period (4-6 h) the CA produced slightly less JH than during the second period, though the difference was not statistically significant.

Growth of reproductive organs and CA, and change in JH biosynthesis in reproductively active adults

In reproductively active long-day adults, the CA, terminal oocytes in females and ectadenia in males grew bigger rapidly after adult emergence (Figs. 26 and 27). At adult emergence, no oocytes were visible in the ovarioles, and the CA were small and produced a very little amount of JH *in vitro*. On day 3 of adult life when oocytes became visible in the ovarioles, the CA started increasing in volume and biosynthesizing JH. On day 6, the first ovulation was observed and a half of females examined on day 9 had

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eggs in their oviducts (5 out of 10 females). In the other half of females terminal oocytes were at or just before the stage of chorion formation. The CA became larger until day 9, and their size changed little thereafter. The activity of JH biosynthesis during days 4-9 was relatively constant and attained a maximum value on day 15, although there was a large variation among individuals. A positive correlation was found between the activity of JH biosynthesis and the size of terminal oocyte, although the correlation coefficient was relatively small (r^2=0.330, df=107, p<0.0001; Fig. 28A). A positive correlation was also found between the CA activity and CA size (r^2=0.278, df=99, p<0.0001; Fig. 28B). It was noted that some large CA were not active in JH biosynthesis.

In males, the size of ectadenia increased rapidly after adult emergence and reached about 5 mm by day 12 (Fig. 27A). Their CA grew bigger as observed in females, but the maximum size for males was smaller than that for females.

Growth of reproductive organs and CA, and change in JH biosynthesis in diapause females

Short-day females had undeveloped ovaries with inactive CA. The CA size did not increase greatly at least during the first 30 days after adult emergence (Fig. 26D-F). In short-day males, the ectadenia remained undeveloped and the CA activity was also very low, ranging from 0 (below the detection limit) to 584 dpm (Data not shown). Females kept under short-day conditions for 120 days showed a large variation in body colour, ranging from grade 1, typically found in reproductively active adults, to grade 5 typical of the colour for diapause adults. The size of oocytes and CA, and CA activity in these females also varied with some correlation with their body colour (Fig. 29).

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Females in body colour grades 4 and 5 had undeveloped ovaries. Their CA were small and had low rates of JH biosynthesis. As body colour became green (grade 1) or closer to green (grades 2 or 3), both oocytes and CA increased in size, and the CA produced more JH.

Effects of nervous transection

After the nervous connections between the brain and CC-CA complex were transected, short-day females developed ovaries to various degrees, depending on the time of dissection (10 or 15 days after transection; Fig. 30). After 10 days of operation, mean oocyte size was significantly larger than that in sham-operated and intact females ($p<0.001$, Tukey HSD test). After 15 days of operation, the oocyte size further increased, and the difference between the values on days 10 and 15 was statistically significant ($p<0.001$, Tukey HSD test). Body colour remained unchanged in most bugs (17 females out of 19) for the first 10 days, but most individuals (9 out of 11) changed from brown to green or intermediate body colour by the 15th day. The CA taken from operated females produced more JH than those from intact or sham-operated females, and the difference became statistically significant by the 15th day of operation. ($p<0.001$ for each, Tukey HSD test). The CA from nerve-transected females remained as small as those from sham controls. The bugs 15 days post-operation had the CA significantly larger than those from intact day 45 females ($p<0.01$, Tukey HSD test), but none of the CA in this experiment reached 4×10^{-3} mm^3, the minimal volume found in long-day females with well-developed ovaries.
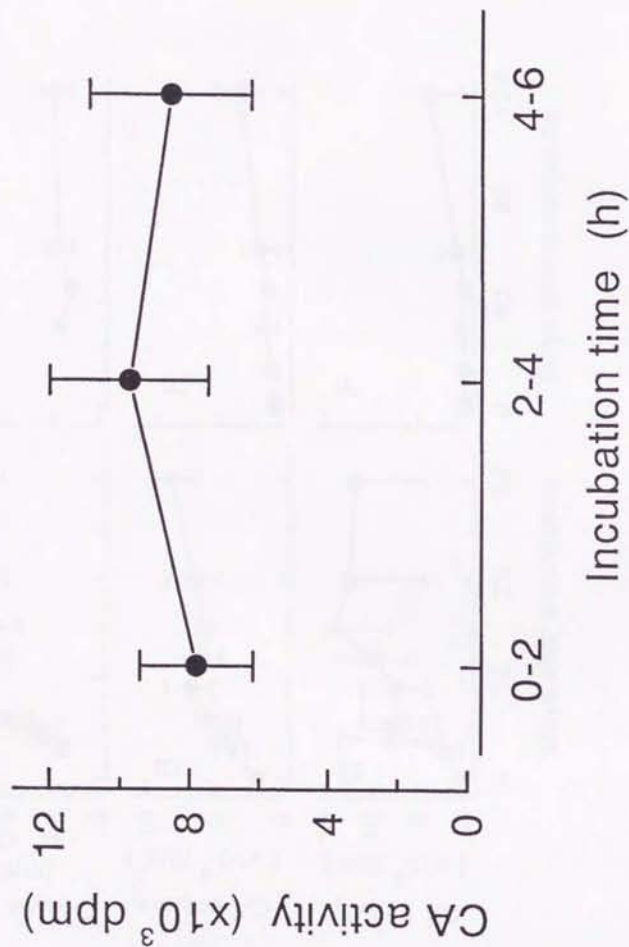
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Fig. 25. Time course analysis of JH-biosynthetic activity by CA taken from reproductively active females of *P. c. stali*. CA were incubated in MEM which was changed every two hours at 30 °C. Amounts of radiolabelled, JH-active product biosynthesized by the CA during each 2-h period were indicated. Each point is the average of 8 determinations with standard deviations indicated by vertical bars.
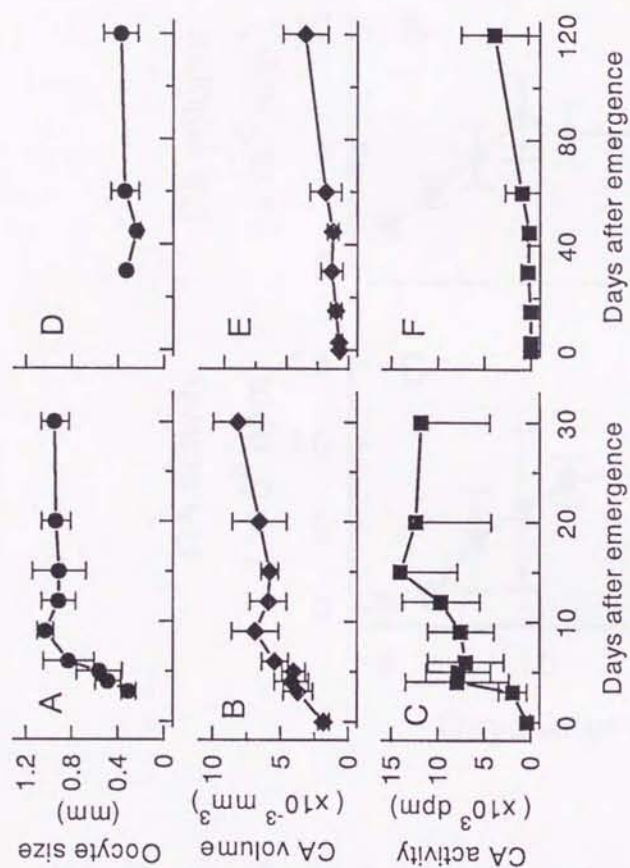
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Fig. 26. Changes in the terminal oocyte size, CA volume and JH-biosynthetic activity by the CA *in vitro* in reproductively active females of *P. c. stali* reared under LD 16:8 h and 25°C (A-C) and diapause female adults reared under LD 12:12 h and 20°C (D-F). Oocyte size (A and D) was represented by the diameter of ovarioles at the position of terminal oocytes, and measurements were started when oocytes were visible at least in one individual in each age group. CA were incubated in MEM for 3 h at 30°C and JH-biosynthetic activity (B and E) was expressed as the amount of radiolabel detected in the JH active fraction after TLC separation of CA products *in vitro*. Each point is the average of at least 10 individuals with standard deviation indicated by vertical bars.
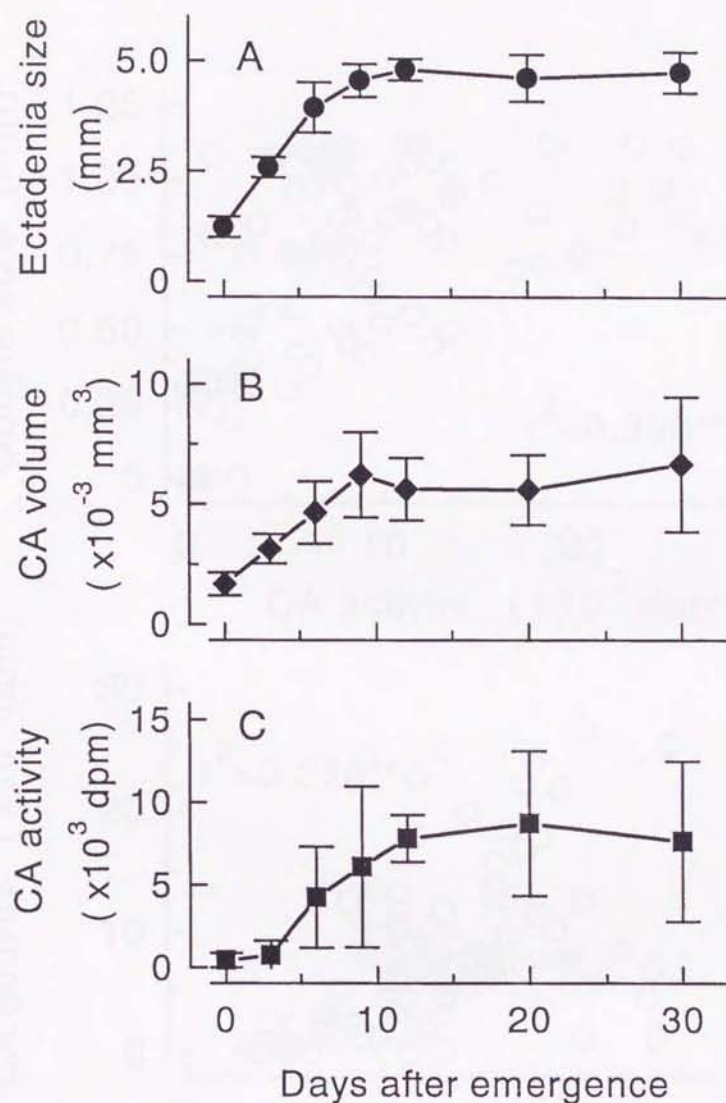
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Fig. 27. Changes in the size of ectodermal accessory reproductive glands (ectadenia; A), volume of CA (B) and JH-biosynthetic activity by the CA *in vitro* (C) in reproductively active males of *P. c. stali* reared under LD 16:8 h and 25°C. Ectadenia size means the width of ectadenia including their reservoir. CA were incubated in MEM for 3 h at 30°C, and JH-biosynthetic activity was expressed as the amount of radiolabel detected in the JH active fraction after TLC separation of CA products *in vitro*. Each point is the average of at least 10 individuals with standard deviation indicated by vertical bars.
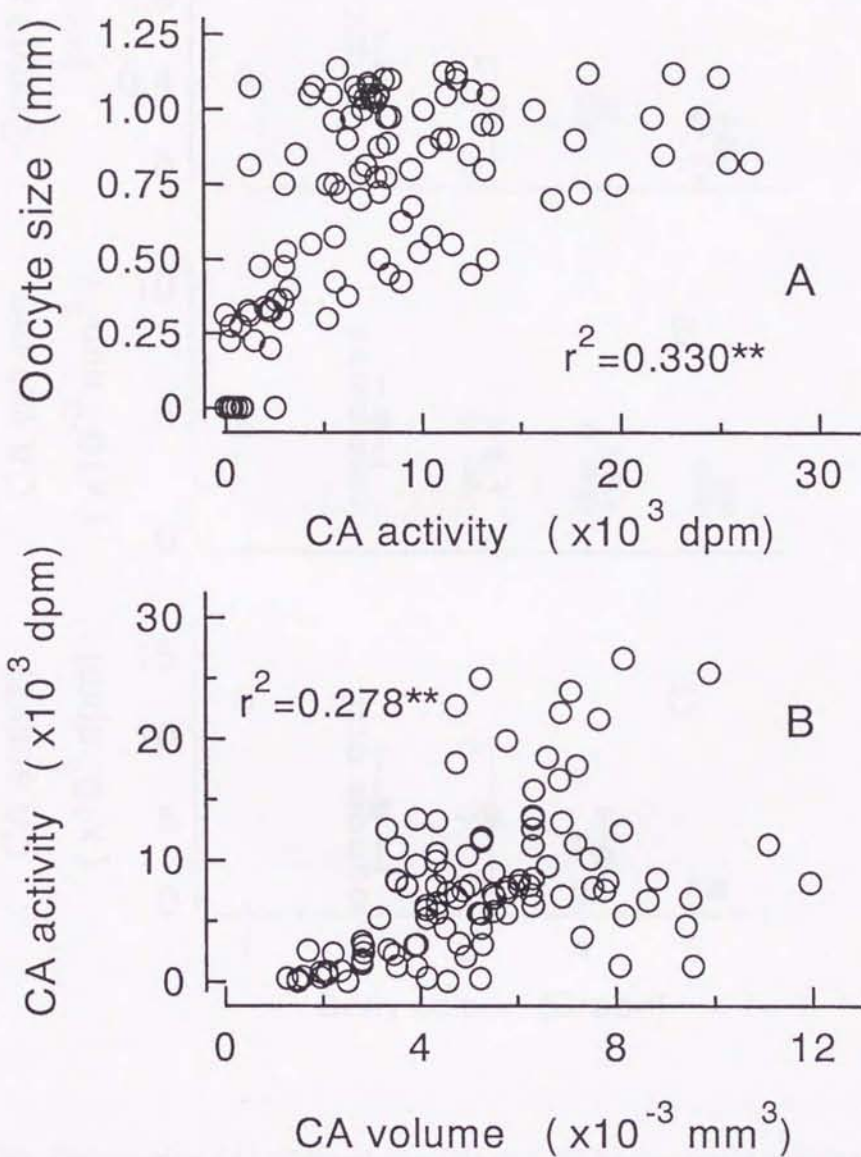
Fig. 28. Relationships between JH-biosynthetic activity and oocyte size (A) and between CA volume and JH-biosynthetic activity (B) in reproductively active females of *P. c. stali*. **: The correlation was statistically significant at p<0.01. For further explanation, see the caption of Fig. 25.
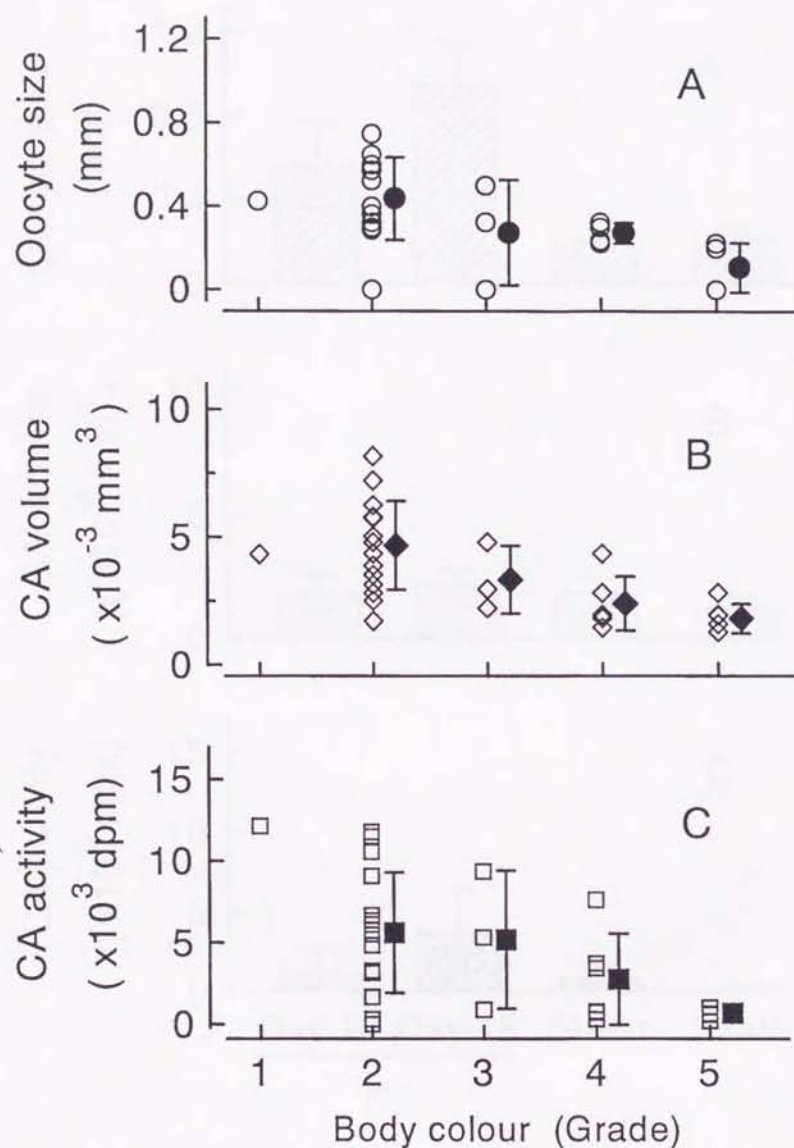
Fig. 29. Oocyte size (A), CA volume (B) and JH-biosynthetic activity (C) as a function of body colour in 120-day-old females of *P. c. stali* kept under LD 12:12 h and 20°C. Results were expressed as the average±standard deviation (closed symbols with vertical bars) along with individual datum points (open symbols). CA were incubated in MEM for 3 h at 30°C, and JH-biosynthetic activity was expressed as the amount of radiolabel detected in the JH active fraction after TLC separation of CA products *in vitro*. For further explanation for body colour grade, see Materials and Methods of Chapter 1.
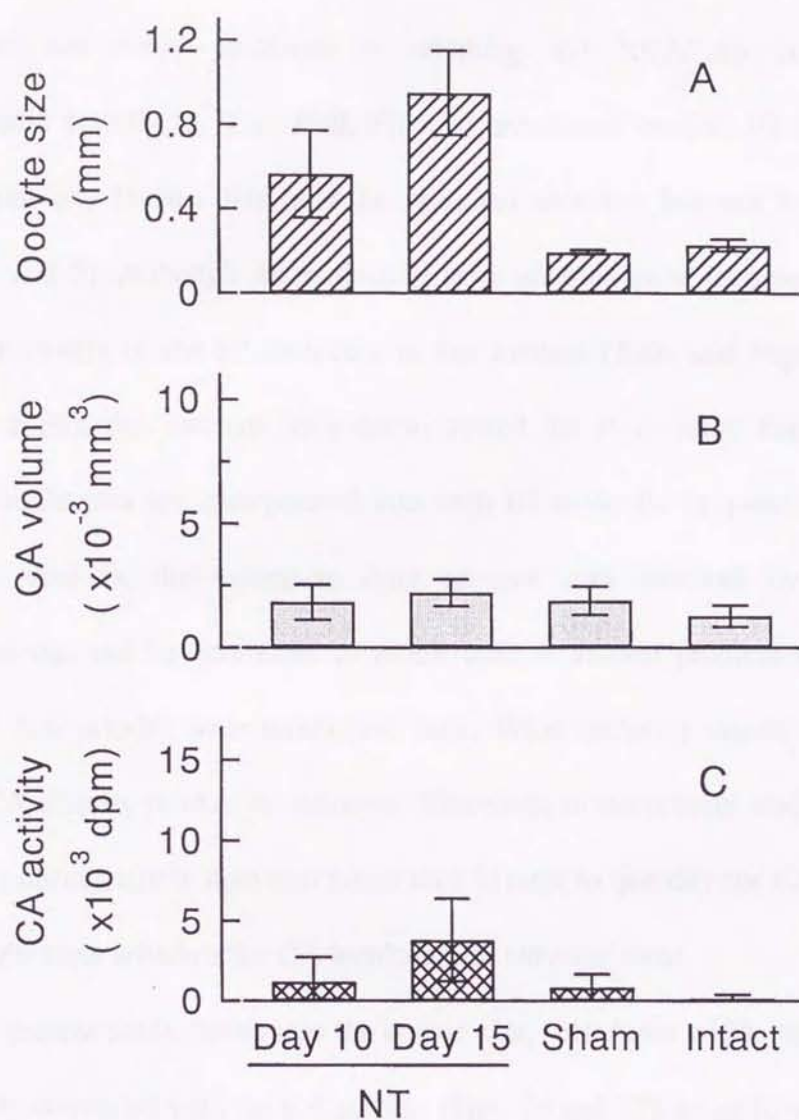
Fig. 30. Oocyte size (A), CA volume (B) and JH-biosynthetic activity (C) in nerve-transected (NT) diapause females of *P. c. stali* dissected either 10 or 15 days after the operation. For a sham operation, the aorta was cut just posterior to the CC-CA complex. Intact: day 45 diapause females without any treatment. CA were incubated in MEM for 3 h at 30°C, and JH-biosynthetic activity was expressed as the amount of radiolabel detected in the JH active fraction after TLC separation of CA products *in vitro*. More than 10 individuals were examined in each treatment, and the average values are indicated. Vertical bars: standard deviation.

Discussion

There are some problems in adopting the RCA for measuring the JH-biosynthetic activity in *P. c. stali*. First, as mentioned earlier, JH in this bug is different from any known JHs, and its chemical structure has not been identified (Chapters 4 and 5). Although the radiolabel from methionine is incorporated into the methyl ester moiety of the JH molecule in this method (Tobe and Feyereisen, 1983; Feyereisen, 1985), this has not been demonstrated for *P. c. stali*. Thus, how many methionine molecules are incorporated into each JH molecule in question can not be determined. That is, the values in dpm or cpm unit obtained by radioactivity measurement can not be converted to mol/h unit. A second problem is the lack of linearity of CA activity over incubation time. What factor(s) causes this temporal change in CA activity *in vitro* is unknown. Therefore, in the present study, I used total amounts of radioactivity in dpm unit rather than in rates to quantify the CA activity, and maintained the time schedule for CA incubation *in vitro* the same.

The present study shows that the oocyte size, ectadenia width, and CA volume are positively correlated with the CA activity (Figs. 26 and 27), as anticipated by results in Chapter 3 based on morphological and surgical techniques. In diapause adults, these correlations disappear if the nervous connections are severed between the brain and CC-CA complex (Chapter 3, Fig 30). These nervous connections are important for the maintenance of diapause (Chapter 3) and the suppression of JH biosynthesis (Fig. 30) under short-day conditions.

In contrast with CA activity, the CA volume in nerve-transected females remains small compared with reproductively active females. The nervous connections appear

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important for a rapid increase in the CA volume, which is typically found in reproductively active adults (Figs. 26 and 27) and also in post-diapause females (Fig. 29). It is likely that large CA can biosynthesize JH at a higher rate, but small CA as found in diapause adults can produce enough amounts of JH to stimulate ovarian development if the connections between the brain and CC-CA are severed. The CA volume and activity are probably regulated independently in *P. c. stali* adults.

In the present study, the difference in CA activity between females 10 days post-operation and sham-operated females was not statistically significant, whereas the difference in oocyte size between the two was statistically significant. This discrepancy may be explained by the injury effect in sham operation. Although the CA size and CA activity in sham females was not statistically different from those of intact females, the average values for the sham females were slightly greater than intact ones, *e.g.*, CA activity for sham controls was about 5 times higher than that for intact females. This may be attributed to injury made by operation, and this effect may cause comparisons between sham and treated less distinct.

The inhibitory role of nervous connections in JH biosynthesis in adult insects is documented in many other species including *P. apterus* (Hodková, 1976, 1977a, 1979), *L. decemlineata* (For a review, Khan, 1988), *D. punctata* (Stay and Tobe, 1977; Rankin and Stay, 1985; as well as humoral pathway, too), *L. migratoria* (Poras *et al.*, 1983; Okuda *et al.*, 1996), *Protophormia terraenovae* (Matsuo *et al.*, 1997) and *R. clavatus* (Morita and Numata, 1997). In *D. punctata*, the nerve-severing leads to an increase in the mitotic activity of CA cells (Chiang *et al.*, 1995) as well as cell size (Chiang *et al.*, 1998). In this species, removing ovaries from adults induces hypertrophy of the CA

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(Johnson *et al.*, 1993). In *O. fasciatus*, Johansson (1958) has reported that nerve-transected females kept under long-day conditions lay as many eggs as intact females do, but their CA remain small. A similar phenomenon has been found in *P. apterus* (Hodková, 1977b). Independence of CA volume and CA activity has been also well documented in many other species (Cassier, 1990). As agents responsible for regulation of JH biosynthesis by the CA, allatotropins and allatostains have been isolated and sequenced (Stay *et al.*, 1994b; Gäde *et al.*, 1997). However, what factor regulates the size of CA is not well known. The CA size is determined by the number of cells and volume of each cell (Tobe and Stay, 1985). How these variables contribute to the change in CA volume and how the brain regulates these traits in *P. c. stali* is to be elucidated in the future.

Females kept under short day conditions for an extended period change their body colour according to their physiological status, and there is a positive correlation between body colour and CA activity (Fig. 28). This may suggest that the body colour reflects the haemolymph JH titre in *P. c. stali*. Since the CA activity is not the only determinant for JH titre (de Korte and Granger, 1996), further studies are necessary to test this hypothesis. A similar change in body colour is known in several other species of Pentatomidae including *N. viridula* (Kiritani and Hokyo, 1970; Hariss *et al.*, 1984), but its regulatory mechanism is poorly understood (Bouthier and Noël, 1991). The present study has provided evidence for the hormonal mechanism of colour change in *P. c. stali*.

Summary

The sizes of terminal oocytes in females and ectadenia in males were positively correlated with the JH-biosynthetic activity by the CA *in vitro*. The CA size and JH-biosynthetic activity were greater in reproductively active bugs than in diapause ones. Some of the females kept under diapause-maintaining conditions for a long period, *i. e.,* 120 days, started growing oocytes and changed body colour from brown to green. Their CA activity and CA volume varied greatly among individuals, and the variation was correlated with oocyte growth and body colour. In diapause adults, the CA activity was enhanced without conspicuous increase in CA size when the nervous connections between the brain and CA were severed. The operated bugs developed their ovaries, and their CA produced more JH *in vitro* than those of sham-operated or intact diapause controls. These results suggest that the JH biosynthesis by CA and the CA size are independently regulated by the central nervous system.

General Discussion

Results obtained in Chapters 1 and 2 imply that the photoperiod perceived in adults stage play an important role for life cycle control in *P. c. stali*. Chapter 1 shows that younger adults of this species are sensitive to diapause-inducing, short-day photoperiods. Because longevity of adults is relatively long in the field, it is likely that some adults of the first generation live until day-length becomes shorter than the critical photoperiod for diapause induction, and as a result, they cease reproduction and enter diapause. That reproductively active adults are sensitive to short-day photoperiods in the laboratory supports this possibility. Once diapause is induced, diapause development in *P. c. stali* proceeds gradually without any specific stimulus, such as an exposure to low temperatures, under diapause-inducing conditions; diapause adults remain sensitive to short-day photoperiods during earlier part of adult life, and as completing diapause development, they become refractory to short-days and start laying eggs. Chapter 2 shows that in a certain period after adult emergence, a low temperature exposure exerts its intensifying effect on diapause. Although it is difficult to interpret this response in relation to life cycle control, one possible explanation for these results is that this may function to avoid untimely development in a year with warmer late autumn preceded by a short cold period. As pointed out by Tauber *et al.* (1986) and Danks (1987), it may be less likely that these responses to the change in photoperiod play an important role in onset of reproduction after overwintering in the field.

Under natural conditions, most females of *P. c. stali* emerging from early September enter diapause in Nagano Pref. (Yanagi and Hagihara, 1980), and in Ibaraki Pref., females collected on host plants in September have undeveloped ovaries (Shiga

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and Moriya, 1989; Moriya, 1995). However, Oda *et al.* (1979) reported that in a warm year, oviposition by *P. c. stali* females was observed until late September in Nara Pref. Day-length of this time of the year is well below the critical photoperiod of about 13.5 h for diapause induction, which was determined in the laboratory (Yanagi and Hagihara, 1980; Ministry of Agriculture, Forestry and Fisheires, 1986). This discrepancy may be explained as a shift of critical photoperiod depending on the temperature, as reported in many insects including *P. apterus* (Numata et al., 1993) and *Graphosoma lineata* (Musolin and Saulich, 1996). A similar mechanism may exist in *P. c. stali* and such a mechanism may enable to construct a flexible life cycle by increasing the number of generations in a warm year.

Thus far, unfortunately, there is little information available on changes in diapause intensity, sensitivity to photoperiod, and effects of temperature on these traits in the field population of *P. c. stali*, except that Yamada *et al.* (1983) reported that overwintering adults collected in the field turned green quickly if they were exposed to 25°C or higher temperatures in the laboratory. To understand the phenology of this economically important insect, it is necessary to accumulate information on interactions between photoperiod and temperature exerting on diapause intensity or diapause development.

Invasion of this species and other stink bugs into orchards occurs during dispersal or movement between reproducing and overwintering sites, or among patchy reproducing sites (Tanaka, 1979; Uchida et al., 1980; Oda, 1980; Moriya, 1995, 1996). It is likely that they tend to fly actively before undergoing and after terminating diapause to enhance movement to and from overwintering sites. Moriya (1995) reported that

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adults collected in the field in August-September showed increased flight activity, compared with those collected in other time of the year, while he failed to detect such an increase in adults reared under short-day conditions in the laboratory. Relationship between diapause and flight behaviour should be analysed further. Another important factor associated with flight activity is hunger level or food availability. As a matter of fact, in the field, adults attracted by males or synthetic aggregation pheromone accumulate less nutrient reserves than those collected at the same time on their host plants (Shiga and Moriya, 1989; Moriya, 1995). This may imply that tendency to be attracted by aggregation pheromone and probably to fly is dependent on or influenced by bug's hunger level. Moriya (1995) suggests that *P. c. stali* is a seed eater and adults of this bug fly into orchards to feed on seeds within fruits, not fruits themselves. Therefore, to predict when and how *P. c. stali* and other bugs fly into orchards, factors affecting flight activity should be elucidated in relation to movement into orchards. Such factors may include not only physiological status of bugs, but also food availability inside and outside orchards, as well as physical environmental conditions.

In studies on adult diapause, oviposition is very often used as a criterion of diapause termination. However, oviposition occurs as a result of morphogenesis which is initiated after diapause is terminated. In other words, oviposition is a consequence of post-diapause development. Besides, this criterion is applicable only to females. In *P. c. stali*, body colour change is closely associated with diapause. When diapause adults are transferred to long-day conditions after or without chilling, there is a positive correlation between the time at which body colour change occurs and the pre-oviposition period. Body colour, therefore, is useful to assess diapause development with emphasis on a

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dynamic aspect of diapause because it is applicable to both sexes without killing the specimen. Indeed, for example, notable differences in patterns of colour change are observed in regimes where bugs were transferred to short-day conditions after chilling (Chapter 2). As mentioned earlier, this may reflect changes in diapause intensity in these bugs. Such changes can not be detected by following oviposition alone.

Brown body colour found among diapause adults appears so cryptic, at least, for human being when overwintering adults are in leaf litter, their overwintering site. However, from what bugs hide themselves is not well known. Ecological significance for colour change in this species should be explored. Changes in chilling tolerance during diapause development or in relation to body colour change are an interesting subject to elucidate. Indeed, Kiritani and Hokyo (1970) have reported that among overwintering adults of *N. viridula*, individuals showing brown body colour less suffer from mortality than green ones. This difference in survivorship may be explained by a difference in body colour-associated chilling tolerance.

The results obtained in Chapter 3 indicate that the hormone released from the CA play a crucial role in the control of diapause in *P. c. stali*. This CA hormone is referred *a priori* as JH in most cases without any problem. In this study, however, the results of Chapter 4 suggest that the *in vitro* CA products are different from any known JH. The bioassay established in Chapter 5 gives an answer to this problem that the *in vitro* CA product has a juvenilizing effect on last instar nymphs of *P. c. stali* and that the hormone is, accordingly, to be called JH. Chapter 6 shows that the rate of biosynthesis of this hormone correlates well with physiological status of bugs: the rate is high in reproductively active insects and low in diapause ones. Therefore, JH is an important

factor in hormonal control of diapause in *P. c. stali*, but this JH is specific to this and probably to other species of Heteroptera.

Although the importance of JH in control of adult diapause is well established, we may need to examine involvement of factors other than JH in diapause control. Very recently, Morita *et al.* (1999) have reported that in adults of *R. clavatus*, allatectomy suppress development of reproductive organs, but allatectomized adults do not accompany some characteristics found in short-day diapause adults, such as a hard cuticle and high lipid content. This may imply that a higher controlling factor plays a role over JH in diapause regulation. Therefore, attempts to ask physiological or biochemical differences between diapause insects and JH-deprived ones, and reason for such differences, if any, may provide further insight into controlling mechanism of diapause.

The present study indicates that the colour change from brown to green always occurs prior to egg laying, irrespective of mode of diapause termination; spontaneous, photoperiodic transfer, CA implantation, topical application of JH III or nervous transection. As mentioned above, a positive correlation is detected between the time occurring body colour change and the pre-oviposition period after transfer to long-day conditions. Thus, body colour change is very closely associated with diapause. More precisely, body colour change is one of symptoms of diapause syndrome. Therefore, it is not surprising that these two phenomena are under the control of JH, as strongly suggested by the results of Chapter 3: allatectomy and extirpation of the CC-CA complexes from reproductively active adults not only suppresses the development of reproductive organs, but also turns operated insects brown. Implantation of these

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complexes into diapause adults induces green body colour as well as the development of reproductive systems. Upon testing topical applications of synthetic JH III to diapause females showing brown colour, a small dose of it causes body colour change to green, but not oviposition, while higher and repeated doses are required to induce oviposition. These, all together, may imply that, the threshold level of JH titre for body colour change is lower than that for development of reproductive organs.

In several species of Pentatomidae including *N. viridula* (Kiritani and Hokyo, 1970; Hariss *et al.*, 1984), the change in body colour to brown, associated with adult diapause or overwintering, is known, but its regulatory mechanism is poorly understood (Bouthier and Noël, 1991). The present study provides evidence for hormonal mechanism of colour change in these stink bugs, and constitute the endocrinological basis for usefulness of the body colour as an indicator of diapause, which is proposed in *P. c. stali* (the present study) and in *N. viridula* (Harris *et al.*, 1984).

Body colour change found in *P. c. stali* is the one called "morphological change" (Bouthier and Noël, 1991). Mode of changing body colour seems complicated, but this is not a result of pigmentation within cuticle layers because cuticle itself taken from both green and brown adults shows the same colour of very pale green or blue, when the epidermal tissue is removed. A preliminary experiment showed that acidic-alcohol extracts of integument taken from brown adults were red or reddish purple in colour when applied on filter paper. Brown body colour, therefore, seems to occur as a result of pigment deposition in epidermal cells or epidermis-associated tissue. Questions what kind of pigment is responsible for brown and green body colour and how the pigment is deposited in or removed from the epidermal tissue during the process of colour change

is to be answered. Nymphs of this bug show far complicated variations in body colour depending on temperature, photoperiod and density (Ministry of Agriculture, Forestry and Fisheries, 1986; Numata and Kobayashi, 1994). Ecological significance and physiological mechanism of colour variation in nymphs would be a good problem to solve.

In the endocrinological or biochemical point of view, the largest problem with the present study and JH-related studies in heteropteran insects is that the chemical identity of JH is not yet settled (Wyatt and Davey, 1996; Davey, 1997). Ironically, JH in *R. prolixus* is not identified yet, although Wigglesworth extensively studied with this insect in his pioneer work and demonstrated the importance of the CA in metamorphosis and reproduction (Wigglesworth, 1985). In 1980s, in an attempt to identify JH in *R. prolixus*, it was indicated that JH-active fraction in this bug is more polar than JH III, but this attempt finally ended up in vain (Pratt, personal communication). Since comprehensive work by Wigglesworth, a pile of studies related to JH have been done using other heteropteran insects. Some of them provide pieces of evidence that support what is suggested in the present study. For example, the CA of *P. apterus* is shown to biosynthesize a product that behave similarly to JH-active product found in *P. c. stali* on the TLC plate (Hodková *et al.*, 1996). Bowers *et al.* (1983) reported that products *in vitro* by the CA of *O. fasciatus* and *N. viridula* was identified as JH III. However, judging from their photograph in their report, a spot with much higher radioactivity than that of JH III spot is evident. They argued this to be a 'metabolite', but the present study with *N. viridula* and other bugs indicates that this 'metabolite' seems to correspond to the product that is commonly found among all these species used in the present study and is

JH-active on *P. c. stali*. Therefore, at least, it is very likely that JH in *P. apterus* and *O. fasciatus* is the same as that in *P. c. stali*. Baker *et al*. (1988) provide a piece of evidence along this line in *O. fasciatus*: they have failed to detect any known JHs in this bug. Davey (1997) have mentioned that, the products by the CA of *P. c. stali* is highly active in his patency assay using ovarian follicle cells of *R. prolixus*. These pieces of information, all together, suggest the presence of JH that is not the same as known JHs and specific to Heteroptera. Elucidating the chemical structure of this JH is, therefore, important and urgent problem to be solved in JH research.

The results of Chapters 4-6 were mainly collected from experiments *in vitro*. It should be kept in mind that results *in vitro* may not reflect the situation *in vivo*. A typical example is reported in *L. migratoria*; in female adult of Japanese strain of this species, there is a negative correlation between the CA activity determined *in vitro* and oocyte size (Okuda *et al*., 1996). Following is suggested to explain this: the CA taken from females bearing less developed ovaries are inhibited via nervous pathway *in vivo*, but if they are excised and brought into incubation conditions *in vitro*, the CA start to produce JH more than they are supposed *in vivo* as a rebound response. Therefore, whether the CA activity determined *in vitro* reflect the JH titre in the haemolymph should be examined carefully.

Recent progress in the research on ecdysteroids also implies that precautions are needed in interpreting results *in vitro* in relation to situations *in vivo*, as follows: The prothoracic glands have been believed to release ecdysone and this inactive form of moulting hormone is to be converted to 20-hydroxy-ecdysone, an active form of moulting hormone. However, at least in Lepidoptera (Warren *et al*., 1988) and several

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species of non-lepidopteran insects (Kiriishi *et al.*, 1990; Oeh *et al.*, 1998), 3-deoxy-ecdysone is indicated to be released from the prothoracic glands. Analyses of products by the prothoracic glands *in vitro* have led to this finding because 3-deoxy-ecdysone is converted to ecdysone in the haemolymph as soon as it is released and it was difficult to detect 3-deoxy-ecdysone in haemolymph samples. Unless such a possibility that the products are converted to an active form after release from the source would be ruled out, precautions for it should be taken in interpreting results obtained *in vitro*. Indeed, in male adults of lepidopteran insects JH acids, not JH is biosynthesized by the CA and acids are sequestered by the accessory reproductive glands and converted to JH in the accessory reproductive glands (Peter *et al.*, 1981).

How the central nervous system controls the JH biosynthesis is to be explained. Allatostatins and allatotropins play a role in controlling mechanism of the JH-biosynthetic activity. These neuropeptides are produced in neurosecretory cells in the brain (Stay *et al.*, 1994b), but mode of action of the neuropeptides is far from full understanding. Transport of allatostatins from neurosecretory cells in the brain to the CA via nervous connections is one possible mechanism for the nervous inhibition of CA activity. In *D. punctata*, Stay *et al.* (1994a) indicate the importance of this pathway. A similar mechanism is suggested for neurally inhibited CA in diapause adults of *L. decemlineata* (Khan and Buma, 1985). In *P. apterus*, nervous connections exert their inhibitory effects on the CA activity in diapause adults (Hodková, 1976, 1977a), while an allatotropic factor is found in extracts of the brain from both reproductively active and diapause adults of this species (Hodková *et al.*, 1996). Although allatostatins and allatotropins are important for regulation of JH-biosynthesis, these neuropeptide are not

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sole player of this process. Many other members, such as ovarian factors, ecdysteroids, JH itself via feed back loop and so on, can be involved (Feyereisen, 1985; Gäde *et al.*, 1997). For *P. c. stali*, extensive study is necessary to elucidate the control mechanism of JH-biosynthesis.

Thus far, a lot of allatostatins are isolated and sequenced from many insects (Stay and Woodhead, 1993; Gäde *et al.*, 1997). Some of them, like callatostatins, however, are not active on the CA of insects from which the peptides in question were isolated, but effective on the CA of another insect. By immunohistochemical technique, alltostatin-like antigen are detected in various parts of insect body, as typically shown in *D. punctata* (Stay *et al.*, 1994b). These facts imply that, when an allatostatic or allatotropic factor is found in a certain tissue, *e. g.*, the brain, it may be premature to conclude that this factor play a regulatory role in JH-biosynthesis. To attain such a conclusion, direct evidence for involvement in regulation of JH-biosynthesis should be put forward. That allatostatin-like antigen are widely distributed within insect body may also suggest multiple functions of allatostatins. Search for roles of allatostatins and allatotropins outside the CA may be of interest.

In intact reproductively active and diapause adults of *P. c. stali*, the size of CA apparently correlates with the JH-biosynthetic activity, but as suggested in Chapter 7, these are regulated independently by the central nervous system. At least two factors, the number of CA cells and cell size are involved in the determination of the CA size. Not many studies have done in the mechanism of CA size regulation except in *D. puctata*. In this species, changes in the CA size, number and volume of CA cells are relatively well documented, and nervous connections and a specific combination of JH and ecdysteriod

titres are suggested to play a role in CA cell proliferation (Chiang *et al.*, 1995). Analyses to understand what causes change in the CA size in *P. c. stali* are necessary to explain regulatory mechanism of the CA growth and activity. When nervous connections are severed in diapause adults, the CA remain small, but they produce enough amounts of JH to stimulate the development of reproductive organs. In this sense, large CA in reproductively active adults seem redundant, but they may need for continuous reproduction or ensuring high reproductive rates under favourable conditions.

It is found that substances known to play a role in the vertebrate have JH-activity, e. g., Némec *et al.* (1993) showed that retinoic acid is JH-active in some insects including *P. apterus* and *D. cingulatus* and thyroids are effective in *L. migratoria* (Davey and Gordon, 1996). The latter authors have listed several similarities between JH and thyroid hormones, and inferred that these similarities may be more than coincidence. It is fascinating, therefore, to speculate that JH is evolutionarily related to vertebrate hormones. To ascertain this, it is necessary to accumulate further knowledge on JH in various insect groups as well as that on its counterparts in organisms belonging to taxonomic groups other than Insecta. Davey and Gordon (1996) have also pointed out the possibility that JH and JH-active substances may have thyroid hormone-like effects on vertebrates as thyroids mimic JH in insects. One has to be very careful to introduce JH-based chemicals as well as any chemicals to the field for controlling pest insects.

The present study examined effects of environmental factors on diapause development and endocrine mode of diapause regulation in *P. c. stali*. Results obtained in this study show that this species has not only many similarities in mechanism of environmental and hormonal control of diapause, but also a peculiarity of JH in this bug

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or probably Heteroptera. Further studies will bring us better understanding on such a peculiarity, which will finally provide comprehensive insight into insect endocrinology beyond a huge range of variety in insects.

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