Chapter 2 Effects of chilling on diapause development

Ambient temperature affects diapause development in various ways depending on the species or the time during the course of diapause development. In some insects undergoing winter diapause, warm conditions which they may encounter during the autumn slow the rate of diapause development (for reviews, Tauber *et al.*, 1986; Danks, 1987; Hodek and Hodková 1988). Chapter 1 shows that diapause of *P. c. stali* can be terminated by transferring insects from short-day to long-day conditions at a constant temperature of 20°C, and that chilling, an exposure to low temperatures is not prerequisite for diapause termination. In this species, however, few studies have been conducted to examine the effects of temperature on diapause termination and related phenomena. In this chapter, following questions are addressed for this species:

Does an exposure to low temperature have any significant impact on diapause termination?

Do bugs under short-day conditions remain sensitive to photoperiods after chilling?

To answer these questions diapause adults were exposed to various low temperatures and their survival rate and body colour were observed. The chilled adults were maintained under either short-day or long-day conditions so that the effects of photoperiod after chilling could also be compared. Results obtained in this chapter suggested that the impact of chilling on diapause and body colour differed depending on the time of exposure to low temperature during the course of diapause development, and that the sensitivity to photoperiod remained after chilling.

Materials and Methods

Insects

A stock culture of *P. c. stali* used in the experiments in this chapter was established with adults collected in a mulberry field in Tsukuba, Ibaraki in 1992 and 1993 and maintained under long-day conditions (LD 16:8 h) at 25°C. In some experiments, insects collected from a different stock colony established in 1994 were used. Insects were fed dry soy beans and peanuts and provided with water supplemented with sodium L-ascorbate (0.05%) and L-cystein (0.025%), and that they were confined in plastic Petri dishes (9 cm diameter, 2 cm height). Newly hatched nymphs were maintained at 25°C under long-day conditions in a large group. After ecdysis to the second nymphal stadium, they were kept in groups of 14 individuals under short-day conditions (LD 12:12 h) at 20±0.5°C to obtain experimental insects. It is reported that all of those kept under short-day conditions from this stadium onward undergo adult diapause (Yanagi and Hagihara, 1980). Newly emerged adults were kept under the same conditions in a group at most 4 individuals per Petri dish.

Chilling

Four experiments were conducted to examine the effects of different temperatures on diapause termination and body colour change. In Experiment I, pairs of day 30-35 male and female adults grown under short-day conditions were either transferred to 15,10 or 5°C, or continuously kept at 20°C for 40 weeks under short-day conditions. In Experiment II, day 30-35 or 60-70 adults were transferred from 20°C to 15,10 or 5°C , or kept continuously at 20°C for 30 or 60 days in short-days. They were

then maintained under long-day conditions at 20°C. Experiment III was performed to determine if effect of an exposure to a particular temperature of 10°C found in Experiment II was age-specific or not. Short-day adults were obtained from the stock colony established in 1994. They were exposed to 10°C or 15°C, or kept at 20° under short-day conditions for 30 days. The exposure to different temperatures started at four different ages of days 20, 30 45 and 60. In Experiment IV, to test whether bugs after chilling remain sensitive to short-day conditions, day 30-35 or 60-70 adults were transferred from 20°C to 15°C or 10°C for 30 or 60 days in short-days. They were then kept under short-day conditions at 20°C. Each pair was checked once a week for oviposition and body colour in Experiment I, and daily in Experiments II - IV.

Results

Experiment I: Diapause termination and colour change at constant temperatures

Figure 5 shows changes in survival rate and body colour after exposure to four temperatures, along with cumulative percentage of ovipositing females at 20°C. Adults at 20-10°C died gradually and their survivorship curves were similar. It took about 30 weeks for half of the bugs to die. At 5°C, on the other hand, bugs died more rapidly and took only 10 weeks to reach 50 % mortality. At 20°C, females started oviposition about 15 weeks after the beginning of experiment and 46 % of them laid eggs during the observation period with a pre-oviposition period of 23.1±4.7 weeks (Average±SD). Fifteen out of 35 females died without egg laying and four did not lay any egg before the end of the observation period. Oviposition occurred only in green females. Mating was sometimes observed while the body colour being checked and all of mating adults were

green. At 15-5°C, neither oviposition nor mating was observed.

At the beginning of the experiment, >80 % of bugs were brown. At 20-10°C, the proportion of brown adults increased slightly during the first 5 weeks, but then declined gradually and most surviving adults eventually turned green. The rate of change in body colour was dependent on the temperature to which insects were treated: the higher the temperature the earlier the change to green. The differences in the time when brown bugs disappeared among these temperature regimes were statistically significant ($P<0.01$, Tukey HSD test). Adults of either body colour died, but mortality was slightly higher in green adults. For example, twenty bugs died at 20°C within 20 weeks after the beginning of experiment, and among them, 12 were green and 8 brown. A similar trend was observed in both 15 and 10°C regimes. At 20°C, a small portion of bugs changed their colour from green to brown again during the last 5 weeks. Adults transferred to 5°C showed no substantial change in body colour. Therefore, results in this experiment confirm that an exposure to low temperatures is not a prerequisite for diapause termination (Chapter 1). Results also show that body colour change can occur even at a of temperature lower than the developmental threshold, 13.8°C (Tanaka, 1979).

Experiment II: Diapause termination and colour change under long-day *conditions after chilling*

The percentage of ovipositing females, that is, percentage of diapause termination varied among regimes (Table 1). When 30-35-day-old adults were exposed to low temperatures, more than 60% of females laid eggs within 60 days of transfer to 20°C and long-day conditions except for those experiencing 5°C, in which 26-35% of

females oviposited. In the regimes where the low temperature exposure was started 60-70 days after emergence, 40-50% of the females laid eggs after a transfer to 20°C and long-day conditions.

Figure 6 shows the pre-oviposition period at 20°C under long-day conditions after chilling. In the regimes where chilling was started on 30-35 days after adult emergence, females exposed to low temperatures for 30 days took longer to start oviposition than those that were exposed for 60 days, and the difference was statistically significant ($P< 0.01$, ANOVA). However, the differences in pre-oviposition period among four temperature regimes in the same time schedule were not statistically significant. These results indicate that the time required for egg laying at the final temperature of 20°C varied with the age of bugs rather than the previous temperature to which bugs had been exposed. On the other hand, day 60-70 bugs took longer to initiate oviposition at the final temperature when they had experienced lower temperature for 30 days. Bugs exposed to 10°C took significantly longer to lay the first eggs after transfer to a long-day photoperiod than those constantly kept at 20°C ($P<0.05$, Tukey HSD test). There was also a significant difference in the pre-oviposition period between the former and the regime with 60 day-exposure to 10°C starting from 30-35 days of adult life ($P<0.05$, Tukey HSD test).

Colour change from brown to green was observed in all regimes. Day 30-35 adults treated at low temperatures for 60 days turned green faster after a transfer to 20°C and long-day conditions than those treated for 30 days. Bugs treated at 10°C for either 30 or 60 days turned green more rapidly than those treated at other temperatures (Figs. 7 and 8). On the other hand, when day 60-70 bugs were treated at low temperatures, a

change in body colour from brown to green at the final temperature of 20°C occurred later as the treatment temperature was lower (Fig. 9). Median values of the time when the brown bugs disappeared were 8, 10 and 14 days in bugs treated at 20, 15 and 10°C, respectively.

Experiment III: Age-dependent change in effects of chilling on diapause intensity

In Experiment II, chilling at a particular temperature of 10°C for 30 days seemed to cause a delay in the time of oviposition when it started from day 60, but not when it started from day 30. To determine this effect was specific to a certain age or such delay gradually became evident as chilling occurred later, four different age groups of bugs were exposed to 15°C or 10°C for 30 days, then transferred to long-day conditions at 20°C, as Experiment II. Bugs derived from a stock colony established in 1994 were used for this experiment.

The percentage of ovipositing females in all regimes in Experiment III under the final conditions of 20°C and long-day photoperiod ranged from 40% to 75%, and in most cases (10 out of 12 regimes), the percentage was higher than 50%.

When chilling was started 20 or 30 days after adult emergence, the pre-oviposition period at the final temperature of 20°C under long-day conditions was not statistically different from those which experienced no chilling (Fig. 10). In the regimes either chilled at 15°C from day 45 or kept at 20°C, then transferred to long-day conditions at 20°C, the pre-oviposition period decreased slightly compared with that of the regimes with chilling starting at younger ages. It took longer for bugs exposed to 10°C from day 45 to lay eggs at the final temperature than those exposed to higher

temperatures from the same age and than those exposed to 10°C from younger ages. The difference in the pre-oviposition period between the 10°C- and 15°C-chilling regimes starting on day 45 was statistically significant ($p<0.05$, Tukey HSD test) although the difference between the former and the regime kept at 20°C continuously was marginal ($0.05<p<0.1$, Tukey HSD test). When chilling at 10°C or 15°C was started on day 60, the pre-oviposition period at the final temperature tended to decrease, compared with that of regimes with chilling starting at younger ages. In the regime kept at 20°C and short-day photoperiod for 90 days (60 days plus 30 days), some females started oviposition before a transfer to long-day conditions. They seemed to terminate diapause by this time. The time of oviposition seem to be related to the time when body colour change took place. More than 75% of females laid eggs within 15 days after they attained body colour grade 3, that is, they showed an intermediate body colour between brown and green (Fig. 11). Therefore, these results suggest that an oviposition-delaying effect specific to 10°C is also age-specific. In this experiment, too, body colour change correlated well with the time of diapause termination, as indicated in Chapter 1.

Experiment IV: Diapause termination and colour change under short-day conditions after chilling

To determine whether bugs remain sensitive to photoperiod after chilling, bugs exposed to low temperatures for either 30 or 60 days were transferred to 20°C and short-day conditions rather than long-day conditions. After a 30-day exposure to 10 or 15°C, no females laid eggs during the observation period of 60 days at 20°C. After 60-day exposure, less than 10% of females started oviposition with a pre-oviposition

period of 24-49 days after transfer to 20°C.

In some bugs that changed their body colour, the rate of change was influenced by temperature (Fig. 12). More than half of the bugs exposed to 10°C for 30 days remained brown even for 60 days after transfer to 20°C. After an exposure to 15°C, it took about 30 days for 50% of bugs to turn green at the final temperature of 20°C under short-day conditions. In the bugs treated at 15°C or 10°C for 60 days, body colour changed more rapidly than in those treated for 30 days, and 50% of bugs developed green colour within 15 and 10 days after exposure to 15°C and 10°C, respectively. In the former five out of 44 adults changed back to brown colour, although Fig. 12 giving only net changes in percentage does not show this observation. After exposure to 10°C, the percentage of brown individuals started increasing in 25 days at 20°C and reached more than 25% at the end of the observation period. Therefore, results in this experiment suggest that diapause adults remain sensitive to short-day photoperiods after chilling.

Table 2. Effect of chilling on oviposition by female adults of *P. c. stali*

Age when chilling started (Days)	Duration of chilling (Days)	Temperature exposed (°C)	Number of insects used	Ovipositing females (%)
30-35	30	20	25	68
		15	29	79
		10	25	60
		5	23	26
30-35	60	20	22	68
		15	22	77
		10	28	78
		5	17	35
60-70	30	20	18	50
		15	17	53
		10	18	41

Diapause females were transferred to 20°C and long-day conditions after an exposure to various temperatures. Date of oviposition was recorded daily for 60 days at the final temperature of 20°C.

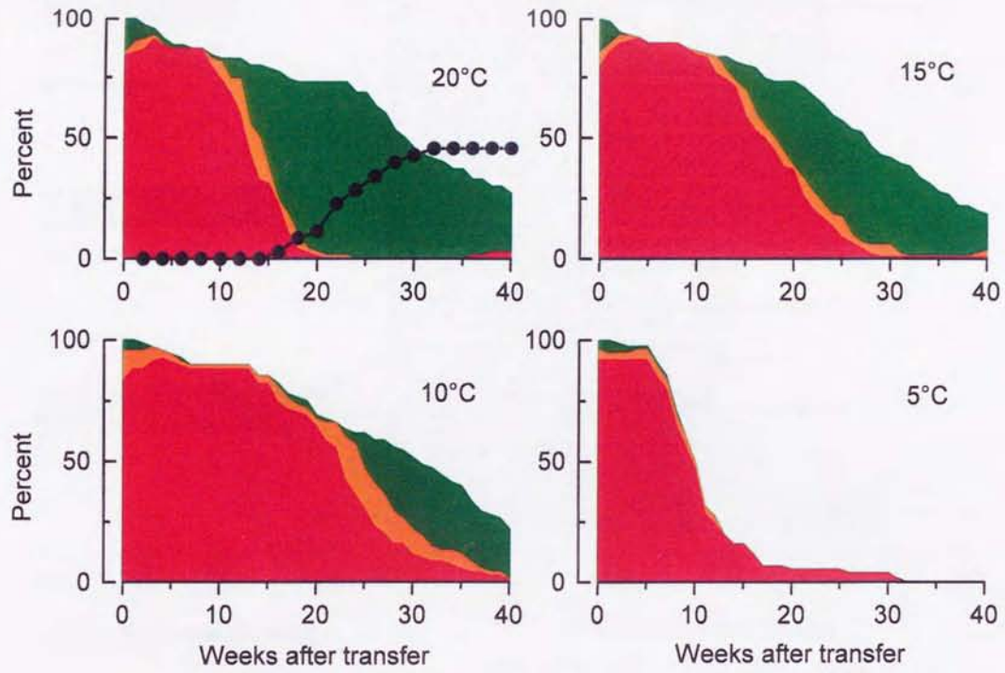

Fig. 5 Colour change and survivorship curves in diapause adults of *P. c. stali* kept at 20-5°C and short-day conditions. Day 30-35 diapause adults reared at 20°C and short-day conditions were transferred to four temperature regimes indicated in each panel. Green, orange and red areas indicate the proportion of bugs with green, intermediate and brown body colour, respectively. Closed circles in the 20°C regime indicate the cumulative percentage of ovipositing females. Each treatment is consisted of 28-43 pairs.

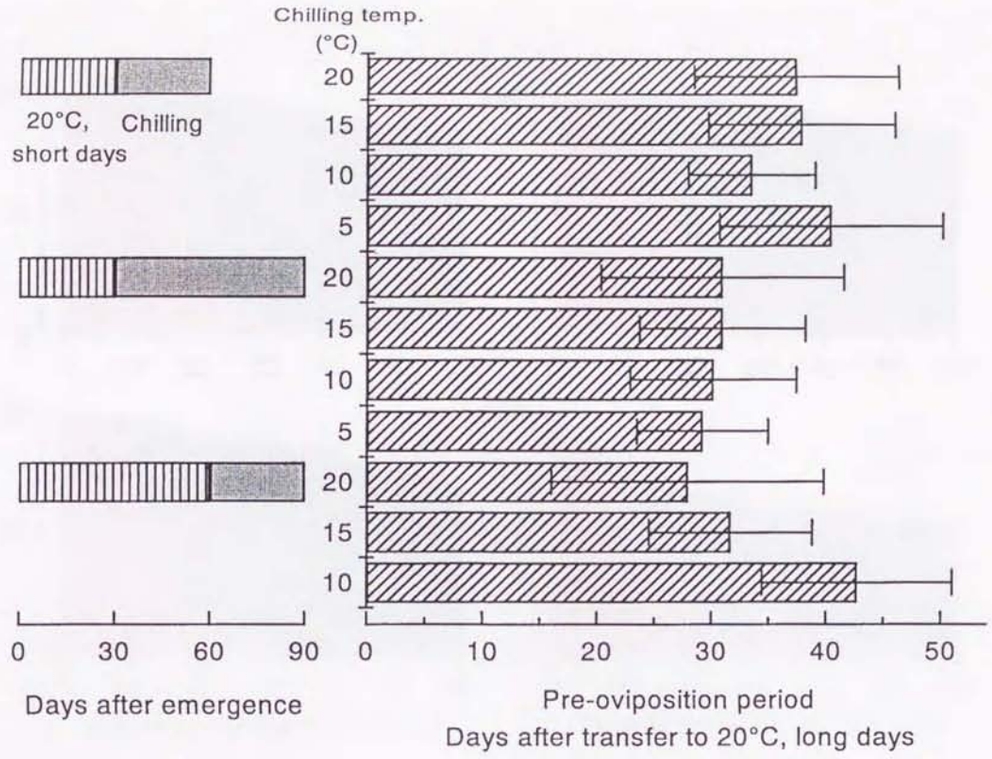

Fig. 6. Pre-oviposition period of *P. c. stali* females exposed to low temperatures. Day 30-35 or 60-70 diapause insects were exposed to various temperatures for 30 or 60 days under short-day conditions, followed by a transfer to 20°C and long-day conditions. In the left of the figure, the time schedules are indicated with hatched and shaded bars showing the period at 20°C under short-days, and chilling, respectively. The chilling temperatures are indicated in the central part of the figure. The right of the figure shows mean values of the time required for the first oviposition at the final temperature of 20°C. Horizontal bars: standard deviation. Each treatment consisted of 17-29 pairs.

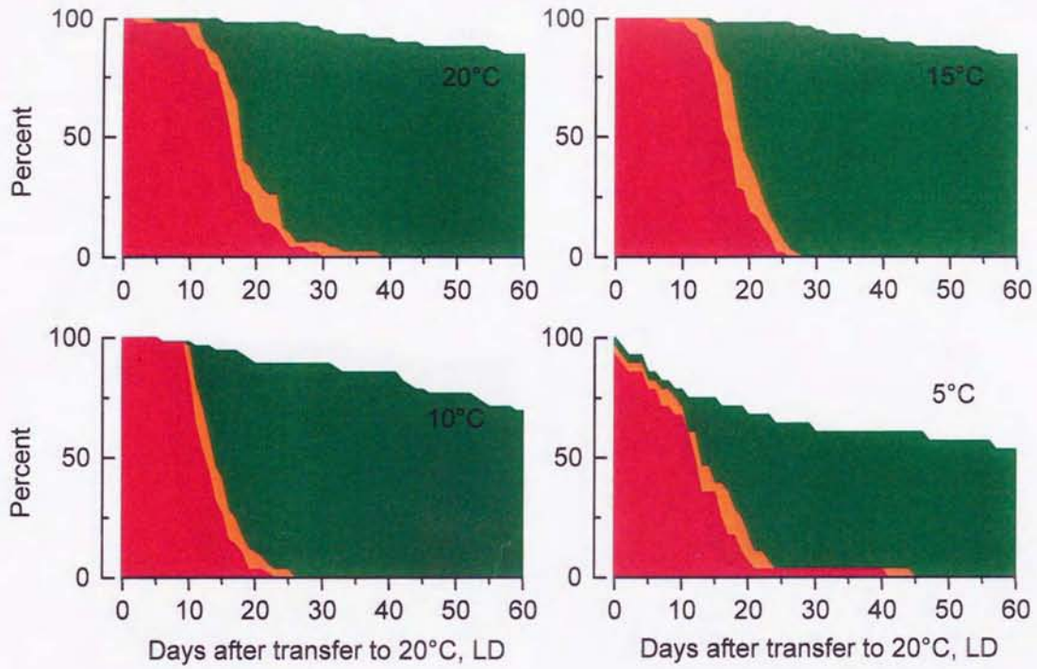

Fig. 7. Colour change and survivorship curves under long-day conditions in *P. c. stali* adults after a 30 day-chilling starting on 30-35 days of adult life. Diapause adults were exposed to a low temperature, which is indicated in each panel, and then transferred to 20°C and long-day conditions. Green, orange and red areas indicate the proportion of bugs with green, intermediate and brown body colour, respectively. Each treatment consisted of 23-29 pairs.

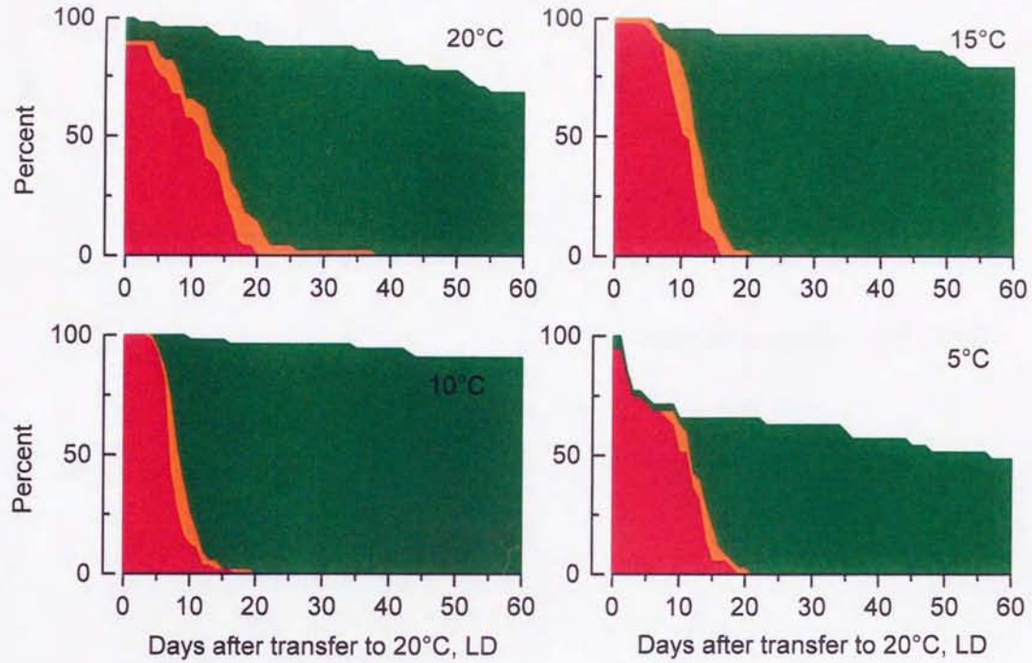

Fig. 8. Colour change and survivorship curves under long-day conditions in *P. c. stali* adults after a 60 day-chilling starting on 30-35 days of adult life. Diapause adults were exposed to a low temperature, which is indicated in each panel, and then transferred to 20°C and long-day conditions. Green, orange and red areas indicate the proportion of bugs with green, intermediate and brown body colour, respectively. Each treatment consisted of 17-27 pairs.

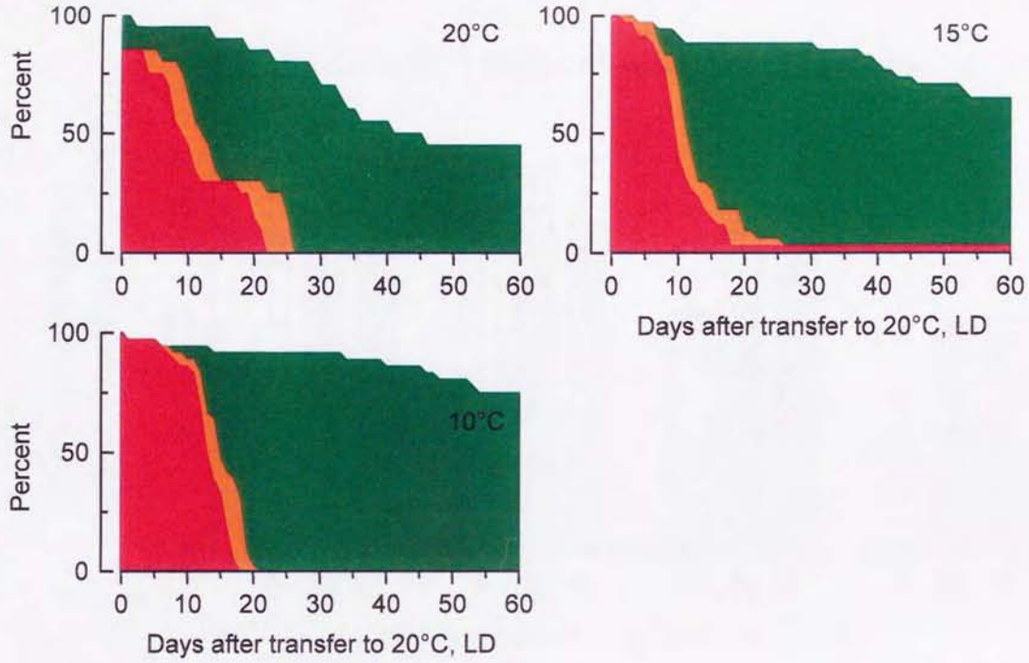

Fig. 9. Colour change and survivorship curves under long-day conditions in *P. c. stali* adults after a 30 day-exposure to 20-10°C starting on 60-70 days of adult life. Diapause adults were exposed to a low temperature, which is indicated in each panel, and then transferred to 20°C and long-day conditions. Green, orange and red areas indicate the proportion of bugs with green, intermediate and brown body colour, respectively. Each treatment consisted of 17-22 pairs.

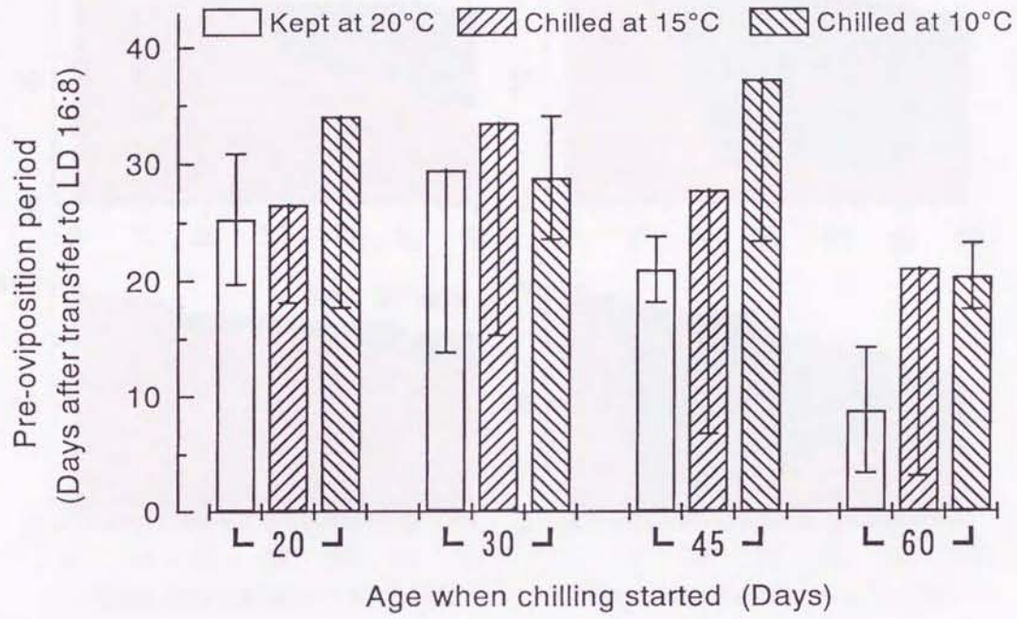

Fig. 10. Pre-oviposition period of *P. c. stali* females after transfer to 20°C and LD 16:8 h. Days 20-60 bugs reared at 20°C and LD 12:12 h were exposed to 20°C, 15°C or 10°C at LD 12:12 h for 30 days, then transferred to the final temperature of 20°C at LD 16:8 h. The average time required to deposit the first egg-batch was plotted against the age (indicated by the numbers along horizontal axis in days after adult emergence) at the beginning of chilling. Each datum was based on 4-10 females. Vertical bars: standard deviation.

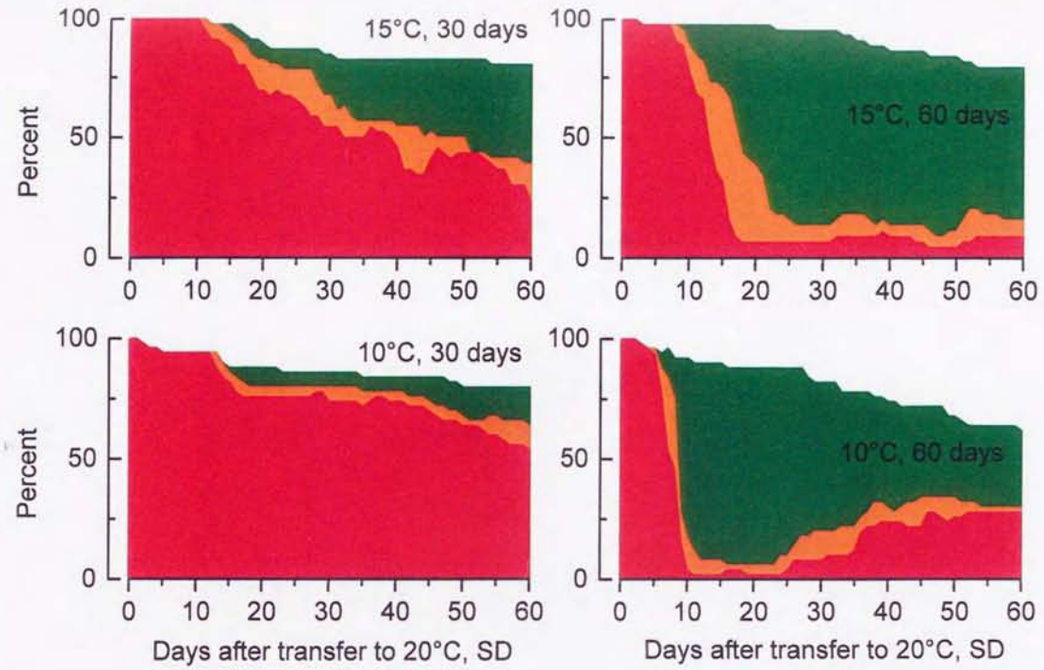

Fig. 12. Colour change and survivorship curves under short-day conditions in *P. c. stali* adults after a 30 or 60 day-exposure to 15°C or 10°C and short-day conditions starting on 35-30 days of adult life. Diapause adults were exposed to a low temperature, which is indicated in each panel along with the duration of the exposure, and then transferred to 20°C and short-day conditions. Green, orange and red areas indicate the proportion of bugs with green, intermediate and brown body colour, respectively. Each treatment consisted of 22-25 pairs.

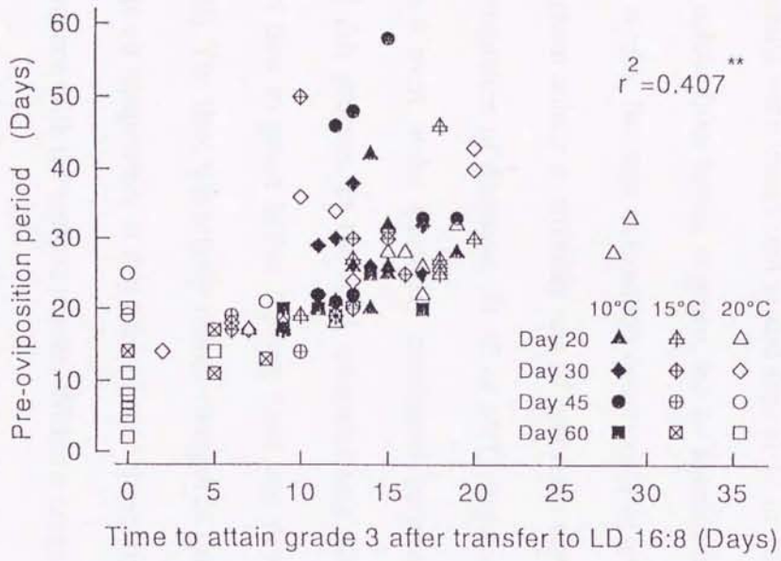

Fig. 11. Relationship between the time required for *P. c. stali* females to attain body colour grade 3 and the pre-oviposition period after transfer to 20°C and LD 16:8 h.

Discussion

Results obtained in the present study indicate that temperature affects the rate of diapause development in a complicated way. In Experiment I, bugs kept at 20°C and a short-day photoperiod started laying eggs after an extended pre-oviposition period (Fig. 5). This suggests that diapause development in *P. c. stali* proceeds gradually without exposure to low temperature, conforming to what was found in Chapter 1. When diapause adults were transferred to and kept at 10 or 15°C, some individuals showed a change in colour from brown to green, but no females from these cohorts laid any eggs within 40 weeks. Because oviposition occurs only in green individuals, a change from brown to green colour is probably one of processes that diapause adults go though for the full termination of diapause. At 10 or 15°C diapause development may proceed to some extent even under short-day conditions because adults at these temperatures completed this process. In the field, overwintering adults in leaf litter are brown in colour and turn to green before leaving there and developing their ovaries in spring (Oda, 1980). The time when body colour changes in the field is around mid April, and the average air temperature at this time of year is usually below 20°C. This observation is in accordance with the present results. Within a range from 10 to 20°C, the lower the temperature at which bugs were kept, the lower the rate of diapause development, on the assumption that body colour change occurs when diapause development proceeds to a certain degree. At 5°C, bugs died more rapidly than at higher temperatures. This is likely related to the fact that bugs were directly transferred from a high temperature to the constant, low temperature without any acclimation.

Experiment II indicates that the 30 day-exposure to low temperatures starting on 30-35 days of adult stage exerted no significant effect on diapause development (Fig. 6).

After a longer exposure to low temperature, the pre-oviposition period is shortened compared with the results after a shorter exposure, but it did not differ statistically among different temperatures in the same time schedule. The latter suggests that diapause development proceeds similarly at different temperatures and appears to conflict with the conclusion from Experiment I that diapause development proceeds more slowly at a low temperature. This is because Experiment I lasted much longer than Experiment II, and the temperature-dependent difference in the rate of diapause development may be manifested in a later time of adult life. Interestingly, in the regimes of 30 day-exposure starting on 60-70 days of adult life, the time of the first oviposition is delayed as the exposure temperature decreases (Fig. 6). This was confirmed by results in Experiment III, but in the latter experiment, the delay was observed in regimes with chilling from day 45 (Fig. 10). Results of Experiment III also suggested that this effect at 10°C is related to a certain age, day 45 in Experiment III, and is not manifested gradually as the time proceeds. These suggest that lower temperatures re-intensify diapause in these regimes. The difference in time when this 10°C-specific effect was detectable between Experiments II and III seems to parallel with that of the time when the first oviposition was observed in regimes kept at 20°C and a short-day photoperiod in the two experiments. This may be attributed to the fact that bugs from different stock cultures were used in these experiments. Another explanation for the 10°C-specific delay in egg deposition is that the rate of post-diapause development at the final temperature is influenced by the previous temperature to which insects were exposed. To test these hypotheses, it is necessary to conduct further experiments where diapause and post-diapause development can be monitored separately. No matter which

hypothesis will be supported, effects of the exposure to low temperatures on diapause development depend on the age at exposure, and it seems that adults in later stages of diapause development are more sensitive to low temperatures than those in earlier stages. In *P. apterus*, Hodek (1978) has described an age-related change in the effect of temperature on diapause: he finds that a low temperature experienced in a later stage of diapause is more effective than an earlier one in terminating diapause under short-day conditions at 25°C.

When cold exposures for 30 or 60 days were followed by transfer to 20°C and short-day conditions, of females from the cohorts of 60-day exposure only a small fraction laid eggs during the observation period. This indicates that bugs remain sensitive to photoperiod after a cold exposure of at least 60 days. In *R. clavatus,* Numata and Hidaka (1984) have reported that diapause adults exposed to 10 or 15°C for 30 days remain sensitive to short-day conditions while those exposed to 15°C or kept at 25°C for a longer period were less sensitive to short-day conditions and the critical day length for diapause termination in those insects was shorter than that in bugs after shorter exposure. In *P. c. stali* how a longer exposure to low temperatures would influence the sensitivity to photoperiod is yet to be examined.

In the regimes where bugs were kept at or returned to 20°C and short-day conditions, some individuals which changed their body colour from brown to green turned brown again. Assuming that colour change occurs when diapause development has proceeded to a certain degree, as mentioned above, the colour changes observed in these regimes suggest that diapause in such adults is re-intensified in response to short-day conditions perceived in the later half of diapause development. If so, in this

bug, during the course of diapause development, there may be a time when diapause is re-intensified under short-day conditions. In *A. acuminata*, Hodek (1979) has reported that short oviposition periods are intervened by periods without oviposition when overwintering adults are transferred to short-day conditions at 25°C. He explains this phenomenon that females of this species that have terminated diapause and started oviposition will become responsive again to photoperiods leading to the cessation of egg laying and this cycle is repeated under short-day conditions. In *R. clavatus* Numata (1987, 1990) has shown that some adults in which diapause was terminated under short-day conditions regain photoperiodic sensitivity and undergo a second diapause in short-day conditions. Ikeda-Kikue and Numata (1992) have also reported that post-diapause adults of *Eurydema rugosa* regain sensitivity to the diapause-inducing photoperiod if they are fed seeds of their host plant, but fail to do so if fed host plant leaves. In *P. c. stali* adults, unlike these species, the time when bugs resume sensitivity to photoperiods is somewhat before ovarian development begins. This means that this phenomenon occurs in the process of diapause development, not after diapause termination. Dietary influence on diapause termination as indicated in *E. rugosa* should be also studied.

Summary

Diapause adults of *P. c. stali* maintained at 20°C under short-day conditions (LD 12:12 h) were exposed to four temperatures of 5-20°C to examine the effect on diapause development which was assessed in terms of oviposition. Diapause adults kept at 20°C under short-day conditions changed their body colour gradually from brown to green and started egg laying after a prolonged pre-oviposition period. Those transferred to

either 10 or 15°C also showed colour change but did not lay eggs. Bugs exposed to 5°C underwent neither body colour change nor oviposition and died more rapidly than those kept at higher temperatures. When 30-day-old diapause adults were chilled at 5, 10 or 15°C for 30 or 60 days and returned to 20°C and long-day conditions (LD 16:8 h), the pre-oviposition period varied primarily depending on the length of chilling period, but not on the temperature. On the other hand, when older diapause adults chilled for 30 days were observed at 20°C and long-day conditions, their pre-oviposition period tended to be longer as the chilling temperature was lower. In this case, a temperature of 10°C appeared to intensify diapause in an age-specific fashion. Therefore, the effect of chilling on diapause development varied depending on the age at which insects were chilled. When chilled bugs were transferred to short-day conditions at 20°C, most females failed to lay any eggs and some turned green, then after a while, some green bugs changed to brown again. These results indicate that bugs remained sensitive to short-day conditions even after a 60-day chilling at 10 or 15°C.

Chapter 3 Hormonal control of diapause and body colour change - Role of the corpus allatum

Adult diapause is characterized by the arrest of reproductive activities. In diapause females, in particular, oviposition and ovarian development are inhibited. However, in diapause males of some species, *e. g.*, *P. apterus* (Zdárek, 1970), it is not the testis but the accessory reproductive glands that are developmentally suppressed. In *N. viridula* (Kiritani and Hokyo, 1970) and *Menida scotti* (Koshiyama *et al.*, 1994), the physiological status of overwintering adults are suggested to be different between the two sexes; females overwinter in a state of diapause while males do so in quiescence with well-developed accessory reproductive glands. In *P. c. stali*, because oviposition is suppressed under short-day conditions (Chapters 1-2), ovaries are very likely to remain undeveloped in diapause females. In male adults, however, no information is available about the physiological status of those kept under short-day conditions in the laboratory. In the first part of this chapter, to characterize physiological aspects of diapause in *P. c stali*, the development of reproductive organs, namely, the ovaries in females and the testes and ectodermal accessory reproductive glands, ectadenia in males were examined.

In many insects, a low haemolymph titre of the gonadotropin, JH is known to induce adult diapause, and the rate of JH biosynthesis by the CA in diapause insects is kept at a very low level. The JH biosynthesis is, in turn, controlled by the brain (de Wilde, 1983; Denlinger, 1985; Wyatt and Davey, 1996). Among heteropteran insects, *P. apterus* is one of the most extensively examined species for hormonal control of diapause (e. g., Sláma, 1964; Hodková, 1976, 1992). In females of this species, a

long-day photoperiod leads to rapid reproduction after adult emergence through increased JH biosynthesis by the CA, while a reduced activity of the CA induces their diapause in response to a short day-length, and the brain exerts its inhibitory effect under short day conditions on the CA activity mainly via nervous pathway. In this chapter, whether a mechanism similar to *P. apterus* is involved in hormonal control of diapause in *P. c. stali* was asked.

The size of the CA is positively correlated with the JH-biosyntetic activity in many insects (Cassier, 1979, 1990; Tobe and Stay, 1985). If so, according to the hypothesis to be tested in this chapter, reproductively active adults will have large CA, and diapause adults small ones. To determine if it is the case in *P. c. stali*, the size of CA from long-day and short-day adults was measured after adult emergence. To know whether deprivation of the source of JH induces physiological conditions similar to diapause in reproductively active adults, the CA was removed from long-day adults. Effects of implantation of the CA into short-day adults were examined to answer the question whether supplementing exogenous JH source could stimulate development of reproductive organs in diapause adults. The possibility for synthetic JH to mimic effects of CA implantation was tested next. Nervous connections between the brain and CA were finally severed in diapause adults to understand the role of brain in diapause control. Because Chapters 1 and 2 show that body colour changes are closely related to diapause in this species, effects of treatments mentioned above on body colour change was also examined.

Materials and Methods

Insects

Adults of *P. c. stali* used in this chapter were obtained from the same stock culture as in Chapter 1. The degree of ovarian development in female adults was graded into six steps according to Hodek (1971) and Saunders (1983); undeveloped ovaries were classified as grade 1 and well-developed ovaries with chorionated oocytes in ovarioles, not ovulated yet, as grade 5. Ovaries with eggs in the oviducts were referred to as grade 6. Grade 2-4 were classified according to the extent of vitellogenesis. At the same time, the diameter of oocytes or eggs at the posterior end of one of the ovarioles was measured with an ocular micrometer. In male adults, the length of the testes was determined. Males have a pair of ectodermal accessory reproductive glands, ectadenia, which are fused at the mid line of the body and accompanied with a reservoir to accumulate the secretions (Pendergrast, 1957). The width of these fused glands including reservoir part across the body axis was measured, and it will be referred to as ectadenia size. Whether the secretory fluid was accumulated in the reservoir of the ectadenia was also recorded. After measurement of testis length, testicular follicles were exposed by rupturing the epithelial sheath with forceps and the proportion of the length of the part occupied by elongated spermatocysts to the whole follicle length was determined by visual observation. Spermatocysts with its length twice or more greater than its width will be called "elongated" ones.

To estimate the volume of CA, the diameters of CA along and across the body axis were measured under a stereo microscope. The CA in *P. c. stali* are fused glands and closely attached to a pair of the corpus cardiacum (CC). They are located posterior

to the brain, under (on the ventral side of) the aorta. The volume of CA was estimated by the following equation by assuming that the shape of CA was a spheroid:

$V=(\pi/6)D_1\times D_2^2$,

where D_1 and D_2 were the diameters along and across the body axis, respectively.

Surgical operations

Extirpation of the CA or corpora cardiaca-corpora allata (CC-CA) complexes was performed with fine forceps through a small incision on the dorsal side of the neck membrane of ether-anaesthetized long-day adults 4 days after adult emergence, when the body wall of bugs was hardened enough for surgical operation and the size of reproductive organs was still relatively small. For allatectomy, the CA removal, these glands were gently pulled by forceps to separate them from the CC. For removing the CC-CA complexes, nervous connections between the brain and CC were severed by gently pulling the complexes by forceps, then they were extirpated along with a small piece of adjacent aorta. For a sham operation of the extirpation of CA or CC-CA complex, the aorta was pinched and slightly lifted up by the forceps. After the operation the wound was treated with a small amount of a mixture of penicillin G and streptomycin. For the implantation experiment, the CC-CA complexes, instead of the CA alone, were used to avoid any damage to the CA. Either two CC-CA complexes (Treatment), or two pieces of aorta (Sham operation) taken from reproductively active females were implanted into ether-anaesthetized adults through a small incision of the fifth sternum. Recipients were short-day adults 30 days after emergence, when diapause in those bugs were deepened and they were brown in body colour, a typical of diapause

bugs (Chapter 1). After implantation, a small amount of mixture of the antibiotics was applied to the wound, which was thereafter sealed with dentist's paraffin. The nervous connections between the brain and CC-CA complexes were severed in a similar way to removal of the CC-CA complexes, but the complexes remained attached to the aorta. For a sham operation only the aorta was cut at the level just behind the CC-CA complex. Nervous transection was performed in short-day adults either 4 or 30 days after emergence, when diapause or diapause syndrome was possibly not fully induced yet, and fully established, respectively (Chapter 1).

To examine whether exogenous synthetic JH exerts an effect on diapause bugs similar to the CA implantation, topical application of JH III was performed on day 30 short-day adults. Synthetic JH III (Sigma) was dissolved in acetone at appropriate concentrations, and an aliquot of 1 µl of these solutions was applied to adult sternites every two days up to either three times or 15 times. Three doses were used: 1, 5 and 25 µg in each application. To control insects 1 µl of acetone was applied.

After surgical operations and JH III application, the body colour and oviposition were recorded daily . Insects were dissected to examine the development of reproductive organs 30 days after treatments. Body colour was assessed as described in Chapter 1.

Results

Development of reproductive organs and volumetric change of corpora allata

In newly emerged females reared under long-day and short-day conditions, ovaries were not developed and classified as grade 1, and oocytes were not seen under a stereo microscope (Fig. 13A and B). In long-day females, oocytes differentiated and

became visible under a stereo microscope by 4 days after adult emergence and, thereafter, ovarian development proceeded. Mature oocytes or eggs were found in the oviducts of day 18 females. The diameter of oocyte (or eggs in well-developed ovaries) and developmental grade of ovary attained maxima of 0.8 mm and 5.9 on days 21 and 18, respectively. On the other hand, ovaries of short-day females remained undeveloped until 30 days after adult emergence.

The length of testes was not significantly different between males reared under long-day and short-day conditions (Fig. 13D). Elongated spermatocysts were observed, irrespective of the photoperiod at which males were reared (Fig. 13E). In contrast, the ectadenia of long-day males developed rapidly after emergence, and a large amount of secretory fluid was accumulated in the reservoir of ectadenia. The ectadenia size attained a maximum on day 21, three times as large as that of day 0 males. The ectadenia of males kept at a short-day photoperiod were less developed than those of males under long-day conditions throughout the observation period, and there was no accumulation of secretory fluid in the reservoir (Fig. 13F).

Under long-day conditions, the CA of both sexes developed to increase by about four times in volume within 30 days after adult emergence. The CA volume in bugs reared under short-day conditions, meanwhile, changed little (Fig. 13C and G). Therefore, the CA size positively correlated with the development of ovaries in females and ectadenia in males.

These results indicated that in long-day females, the ovaries developed rapidly after adults emergence whereas in short-day insects the ovarian development was suppressed. In male adults, ectadenia, not testes, developed rapidly under a long-day

photoperiod, and they remained undeveloped under short-day conditions as the ovaries in females. Therefore, short-day male adults are likely to enter diapause as females reared under short-day conditions, and the developmental status of ovaries and ectadenia can be used as an indicator of diapause. In the following experiments, effects of various treatments were assessed in terms of the grade of ovarian development and the size of ectadenia, as well as the body colour grade.

Extirpation and implantation of the CA or CC-CA complexes

Large CA in long-day bugs may be related to active JH biosynthesis (Cassier, 1970, 1993, Tobe and Stay, 1985). To elucidate the role of CA in the rapid development of reproductive organs under long-day conditions, the CA or CC-CA complex were extirpated from day 4 females kept under long-day conditions. Thirty days after extirpation, most allatectomized or CC-CA-extirpated females had undeveloped ovaries (Table 2); the grade of ovarian development was 1.4 and 1.0 in allatectomized and CC-CA-extirpated females, respectively. In addition, in about a half of those females, the body colour turned brown. The average body colour grade 30 days after treatment was 3.5 in allatectomized females and 4.3 in CC-CA complex-removed females. In contrast, the ovaries of sham-operated females developed and they remained green in colour until the end of observation period.

In males allatectomized on day 4 under long-day conditions, the development of ectadenia was inhibited, and no accumulation of secretory fluid was observed (Table 3). More than half of them turned brown. The sham operation showed no inhibitory effect on the ectadenia development and caused no body colour change. Allatectomy did not

seem to affect the testis size and spermatogenesis (Data not shown).

These results indicate that the CA is necessary for rapid development of ovaries and ectadenia in long-day insects, and that by removing the CA, a physiological condition similar to diapause can be induced in long-day bugs.

To test whether the CA implantation stimulate diapause adults to develop reproductive organs and to change their colour from brown to green, the CC-CA complexes were implanted into day 30 short-day adults. A half of the implanted females deposited eggs with an average pre-oviposition period of 18.6 days after the implantation (Table 4). Until 30 days after the implantation, nine out of 10 treated females turned their body colour green. They had well-developed ovaries even under short-day conditions. Sham-operated females remained brown in colour and had undeveloped ovaries at the end of experimental period. In males, the CA implantation also induced the development of ectadenia and accumulation of secretory fluid in the reservoir of ectadenia (Table 5). More than half of the implanted males turned green after the treatment. Sham-operated males revealed neither change in body colour nor the development of ectadenia. The testis size and spermatogenesis was hardly affected by the CA implantation (Data not shown). These results indicates that the CA implantation into diapause adults seemed to induce a physiological condition similar to that of reproductively active insects.

Topical application of JH III

To test whether the effects of the CA implantation on short-day bugs can be attributed to the effects of JH, synthetic JH III was topically applied to diapause adults.

In the regimes where female bugs were received JH III application three times, those treated with the highest dose (25 μg for each application) of JH III turned from brown to green in colour (Table 6). Oviposition was observed only in females treated with the highest dose up to 15 times (25 μg for each application) of JH III. Females treated with lower JH doses 15 times did not deposit eggs, but developed their ovaries in a dose-dependent manner. Control females treated with 1 μl of acetone 15 times had undeveloped ovaries and remained brown in colour. In males, the treatment with JH III not only induced body colour change from brown to green and, but also caused the ectadenia to develop and accumulate the secretory fluid in the reservoir (Table 7). These results indicated that topically applied JH exerted effects similar to those of CA implantation on short-day adults. However, the dose required to develop reproductive organs seemed relatively high.

Transection of nervous connections between brain and CC-CA complex

Extirpation and implantation of the CA suggest that the CA are active in long-day bugs and less active in short-day ones in JH production. In some insects, *e. g.*, *P. apterus* (Hodková, 1976, 1977a) and *L. decemlineata* (Khan, 1988), the CA activity is inhibited via nervous pathway under diapause inducing conditions. To examine if it is the case in *P. c. stali*, nervous connections between the brain and CC-CA complex were transected so that inhibition through the nerve cords, if any, is lifted. When the connections in day 4 or day 30 short-day females were transected, more than two thirds of them started laying eggs within 30 days after the operation (Table 8). All denerved females developed their ovaries and 75 % of them were green in body colour.

Sham-operated females had undeveloped ovaries and remained brown in colour. Nervous transection in males also induced development of ectadenia, accumulation of secretory fluid in the reservoir and body colour change while sham-operated males did not develop their ectadenia nor turned green (Table 9). No statistically significant differences in the CA volume were detected between nerve-severed and sham-operated bugs of either sex. These results indicated that the CA started releasing JH enough to stimulate the development of reproductive organs even under short-day conditions if the nervous connection between the brain and CC-CA complex was transected. This operation, however, seemed to exert little influence on the CA size.

Table 2. Effect of extirpation of the CA or CC-CA complexes on ovarian development and body colour in *P. c. stali* females reared under long-day conditions

Treatment	No. of insects used	Body colour[a)]		Ovarian development	
		Average grade	No. of insects with brown colour	Average grade	No. of insects with undeveloped ovaries
-CA	16	3.5	7	1.4	14
-CC-CA	7	4.3	5	1.0	7
Sham	18	1.3	0	4.3	3

The CA or CC-CA complexes were extirpated from day 4 females, and the insects were kept under long-day conditions for 30 days.

a) Body colour determined at the end of experiment.

Table 3. Effect of extirpation of the CC-CA complexes on development of ectadenia and body colour in *P. c. stali* males reared under long-day conditions

Treatment	No. of insects used	Body colour[a)]		Development of ectadenia	
		Average grade	No. of insects with brown colour	Width[b)] (Average±SD)	Fluid[c)]
-CA	7	3.7	4	2.56±0.35	-
Sham	10	1.8	0	4.66±0.62	+

The CA or CC-CA complexes were extirpated from day 4 males, and the insects were kept under long-day conditions for 30 days.

a) Body colour determined at the end of experiment.

b) The major diameter of ectadenia was measured.

c) Reservoir of the ectadenia was filled with fluid (+), or no fluid (-).

Table 4. Effect of CC-CA implantation on ovarian development, oviposition body colour and body colour in *P. c. stali* females reared under short-day conditions

Treatment	No. of insects used	Body colour (Average grade)		Ovarian development (Average grade)	No. of females laying eggs	Pre-oviposition period after implantation (Average±SD)[a)]
		Day 30	Day 60			
+2 CC-CA	10	4.9	1.8	5.8	5	18.5±0.5
Sham	12	4.8	4.5	1.3	0	-

Two CC-CA complexes taken from reproductively active females were implanted into day 30 diapause females, and the insects were kept under short-day conditions for 30 days before dissection. Sham: two pieces of aorta were implanted.

a) Days after implantation.

Table 5. Effect of CC-CA implantation on development of ectadenia and body colour in *P. c. stali* males reared under short-day conditions

Treatment	No. of insects used	Body colour (Average grade)		Development of ectadenia	
		Day 30	Day 60	Width[a] (mm) (Average±SD)	Fluid[b]
+2 CC-CA	9	4.7	2.7	3.90±0.82	+
Sham	6	4.8	5	2.46±0.76	-

Two CC-CA complexes taken from reproductively active females were implanted into day 30 diapause males, and the insects were kept under short-day conditions for 30 days before dissection. Sham: two pieces of aorta were implanted.

a) Major diameter was measured.

b) Reservoir of the ectadenia was filled with fluid (+), or no fluid (-).

Table 6. Effect of JH III on ovarian development, oviposition and body colour in *P. c. stali* females reared under short-day conditions

Dose (μg)		No. of insects used	Body colour[a] (Average grade)	Ovarian development (Average grade)	No. of females laying eggs	Pre-oviposition period (Average±SD)[b]
1		7	4.0	N.D.[c]	0	-
5	×3	10	3.9	N.D.	0	-
25		10	2.2	N.D.	0	-
0		9	3.9	N.D.	0	-
1		8	2.5	2.3	0	-
5	×15	7	1.6	3.3	0	-
25		5	2.0	5.8	5	24.8±3.3
0		9	4.2	1.7	0	-

JH III dissolved in acetone was topically applied to day 30 diapause females three times (×3) or 15 times (×15) every two days, and the insects were observed for oviposition for 30 days and dissected at the end of observation period.

a) Body colour determined at the end of experiment.

b) Days from the beginning of JH applications.

c) Not determined.

Table 7. Effect of JH III on development of ectadenia and body colour in *P. c. stali* males reared under short-day conditions

Dose (μg)		No. of insects used	Body colour[a] (Average grade)	Development of ectadenia	
				Width[b] (mm) (Average grade)	Fluid[c]
1		5	2.8	2.60±0.26	+, -[d]
5	×15	9	2.0	3.88±0.63	+
25		11	1.8	4.30±0.20	+
0		10	3.8	2.87±0.62	+, -[e]

JH III dissolved in acetone was topically applied to day 30 diapause males 15 times every two days, and the insects were kept under short-day conditions for 30 days.

a) Body colour determined at the end of experiment.

b) Major diameter was measured.

c) Reservoir of the ectadenia was filled with fluid (+), or no fluid (-).

d) Two males showed + and three showed -.

e) Three males showed + and seven showed -.

Table 8. Effect of nervous transection between the brain and CC-CA complex on ovarian development, oviposition and body colour in *P. c. stali* females

Treatment	Age at treatment (Days)	No. of insects used	Body colour[a]		Ovarian development (Average grade)	No. of females laying eggs	Pre-oviposition period after transection (Average±SD)[b]	CA volume ($\times 10^{-3}$ mm^3) (Average±SD)	
			Average grade	No. of insects with green colour					
NT	4	12	2.3	9	6.0	8	22.3±4.3	1.42±0.39	NS[c]
Sham		10	3.9	1	1.0	0	-	1.73±0.30	
NT	30	5	2.6	4	5.0	4	25.0±5.3	1.18±0.32	NS[c]
Sham		5	4.2	0	1.0	0	-	1.20±0.23	

Nervous connections between the brain and CC-CA complex (NT) or aorta (Sham) of diapause females were transected, and the insects were kept under short-day conditions, observed for oviposition for 30 days and dissected at the end of observation period.

a) Body colour determined at the end of experiment.

b) Days after nervous transection.

c) The difference was not statistically significant between NT and sham regimes at the same age.

Table 9. Effect of nervous transection between the brain and CC-CA complex on development of ectadenia body and colour in *P. c. stali* males

Treatment	Age at treatment (Days)	No. of insects used	Body colour[a)]		Development of ectadenia		CA volume ($\times 10^{-3}$ mm^3) (Average±SD)	
			Average grade	No. of insects with green colour	Width[b)] (mm) (Average±SD)	Fluid[c)]		
NT	4	9	2.4	7	4.58±0.58	+	1.48±0.42	NS[d)]
Sham		6	4.3	0	2.68±0.67	-	1.34±0.52	
NT	30	11	2.0	11	4.66±0.50	+	1.95±0.91	NS[b)]
Sham		5	3.4	1	3.34±0.94	+, -	1.98±0.71	

Nervous connections between the brain and CC-CA complex (NT) or aorta (Sham) of diapause males were transected, and the insects were kept under short-day conditions, and dissected 30 days after nervous transection.

a) Body colour determined at the end of experiment.

b) Days after nervous transection.

c) Reservoir of the ectadenia was filled with fluid (+), or no fluid (-).

d) The difference was not statistically significant between NT and sham regimes at the same age.

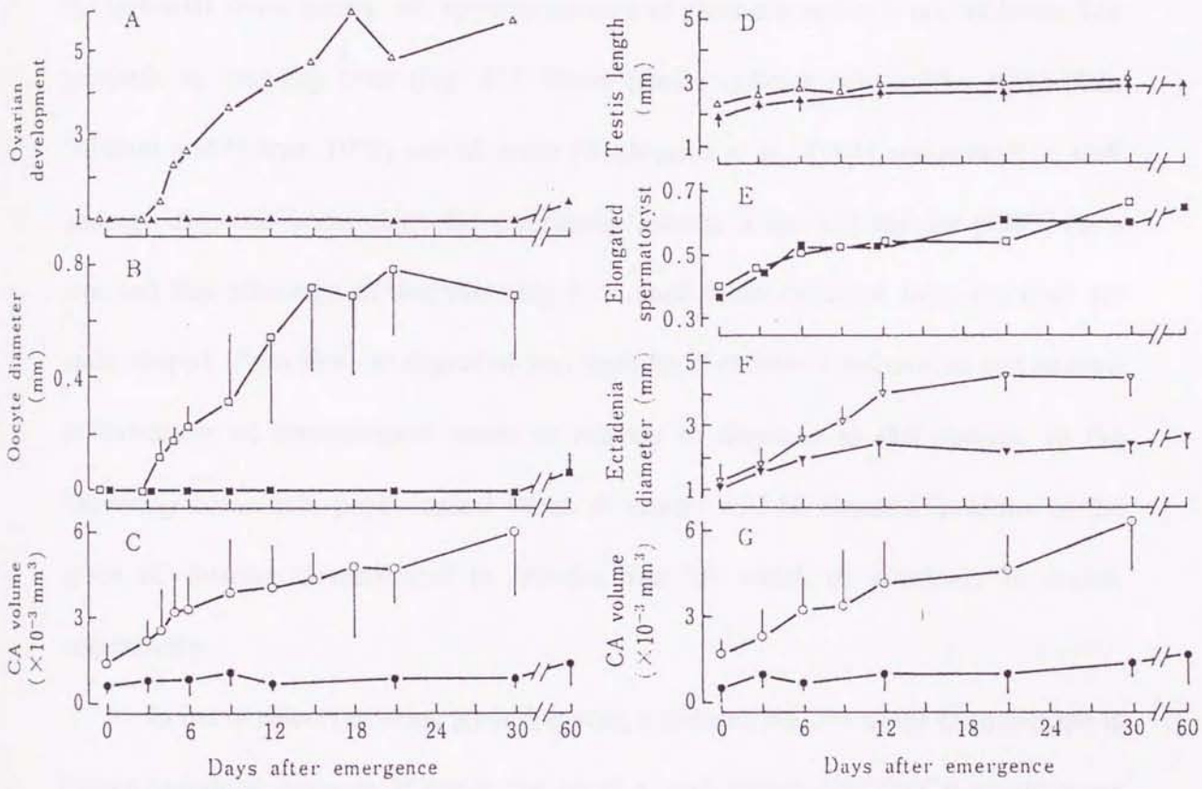

Fig. 13. Development of the ovaries, testes, ectadenia and CA in *P. c. stali* females (A-C) and males (D-G) reared under long-day (open symbols) and short-day (solid symbols) conditions at 20°C. Each point was based on 5-10 individuals and vertical bars indicate standard deviation. A: grade of ovarian development, B: oocyte diameter, C: CA volume in females, D: testis length, E: proportion of the elongated spermatocysts in the testicular follicles, F: width of ectadenia, and G: CA volume in males.

Discussion

In females of *P. c. stali*, as anticipated by the results obtained in Chapters 1-2, ovaries develop rapidly after adult emergence under long-day conditions, whereas the ovarian development is halted under short-day conditions. In short-day males, the development of ectadenia is inhibited as that of ovaries in short-day females. In contrast, the testicular development and spermatogenesis of short-day males is not inhibited, but proceeds as long-day ones (Fig. 13). These results indicate that, unlike *N. viridula* (Kiritani and Hokyo, 1970) and *M. scotti* (Koshiyama *et al.*, 1994), males of *P. c. stali* undergo diapause under short-day conditions. Indeed, Shiga and Moriya (1989) have reported that ectadenia of overwintering *P. c. stali* males collected from the field are undeveloped. Therefore, the degree of development of ectadenia and ovaries can be used as indicators of physiological status in relation to diapause in this species. In the following discussion, physiological status of insects will be assessed in terms of the grade of ovarian development in females and the width of ectadenia in males, respectively.

In many insects entering adult diapause, a reduced JH titre in the haemolymph is known to induce diapause. If this is true for *P. c. stali*, removal of the CA would cause long-day bugs to show a physiological condition similar to diapause. The present results support this hypothesis; removal of the CA or CC-CA complex inhibited the development of reproductive organs in females and males and also induced brown body colour (Tables 2 and 3). Deprivation of the CA, thus, causes operated insects a physiological condition similar to diapause. On the other hand, the implantation of extra CC-CA complexes into diapause adults induced ovarian development and

oviposition in females and development of ectadenia in males after their body colour turned green (Tables 4 and 5). This operation apparently causes rapid development even under diapause-maintaining conditions. Topically applied synthetic JH III also induced the development of reproductive organs and turned body colour into green in both sexes (Tables 6 and 7). This effect is comparable with that of the CC-CA implantation into diapause bugs. Therefore, the effect of CC-CA implantation can be attributed to that of exogenous JH. These results indicate that JH plays a crucial role in the hormonal control of adult diapause, as well as body colour change in *P. c. stali*. A similar controlling mechanism is suggested in several heteropteran insects including *P. apterus* (Sláma, 1964; Hodková, 1976, 1977a), *O. fasciatus* (Rankin and Riddiford, 1977, 1978), *D. baccarum* (Conradi-Larsen and Sømme, 1978) and *R. clavatus* (Numata and Hidaka, 1984; Morita and Numata, 1997).

The transection of nervous connections between the brain and CC-CA complex showed diapause-terminating effect, as did the implantation of CC-CA complexes and JH application (Tables 8 and 9). This indicates that the brain exerts an inhibitory effect on the CA via nervous pathway in diapause bugs, and that, if deprived of this inhibition, the CA begins to release enough amount of JH to stimulate development of reproductive organs. This also implies that any humoral factor does not play an important role to inhibit the CA activity in diapause insects. A similar nervous inhibition is known in *O. fasciatus* (Johansson, 1958) and *P. apterus* (Hodková, 1976, 1977a, b). In *L. decemlineata*, Kahn and Buma (1985) have suggested that the CA of diapause adults is inactivated by an inhibitory substance which is transported through axons and released from the neurosecretory synapses in the CA. In the last species, however, humoral

factors have also been suggested to play a role in control of CA activity (de Wilde and de Boer, 1969). The origin of inhibitory effects within the brain has been shown to be neurosecretory cells in pars intercerebralis in *P. apterus* (Hodková, 1976, 1977a, b, 1979) and those in pars lateralis in *L. decemlineata* (Kahn et al., 1986), respectively. In *P. c. stali*, mode of the CA regulation by the brain is to be determined.

In *P. c. stali*, the extirpation of CC-CA complexes appears to turn the operated insects brown more effectively than the allatectomy. This implies that some supplemental factor(s) released from the CC may be involved in the control of body colour. In *P. apterus*, Sláma (1964) has shown that the lack of 'activation hormone' released from the CC, not JH, is responsible for very low respiratory rates characteristic to diapause females. In *R. clavatus*, females with CC-CA complex extirpated tend to retain eggs in their oviducts after the operation while allatectomized females do not (Morita and Numata, 1997). This may indicate that a factor from the CC would modify the movement of oviducts. Very recently, Morita *et al.* (1999) have reported that allatectomized adults of *R. clavatus* have a soft cuticle and low lipid content, which are characteristics of reproductively active bugs, in contrast to a hard cuticle and high lipid content in diapause ones. This may imply the presence of a humoral factor functioning at a higher level over JH in diapause control. Therefore, the role of CC and other factors in control mechanism of diapause should be carefully examined in the future.

Results obtained in this chapter show that body colour change from brown to green always precedes oviposition in females which are implanted with the CC-CA complexes, nerve-transected or topically applied with JH III. This may suggest that body colour change occurs rapidly than oviposition. These results also implies that epidermis

are more sensitive to JH than ovaries, because more doses are required for inducing oviposition than those for changing body colour.

As mentioned above, the CA of long-day bugs are active in JH production, but those of short-day bugs not. The CA of long-day bugs increase their size rapidly after adult emergence while in short-day adults, the CA volume is not greatly changed throughout the observation period. In intact insects, therefore, there is a positive correlation between the CA volume and CA activity. This correlation, however, appears to be diminished by cutting nervous connections, because in nerve-transected bugs, the CA remain small, but they seems to secrete sufficient amounts of JH to terminate or prevent diapause. Further analyses will be done with respect to the relationship between CA volume and JH-biosynthesis in Chapter 6.

Summary

After allatectomy from reproductively active adults of *P. c. stali*, development of ovaries in females and ectadenia in males was inhibited and their body colour changed from green to brown. Implantation of CC-CA complexes into diapause adults induced development of ovaries and ectadenia, and green body colour. Topical application of synthetic JH III and nervous transection between the brain and CA exerted effects similar to CC-CA implantation on diapause adults. These results indicate that JH plays a crucial role in control of adult diapause in *P. c. stali* and the brain inhibits production of JH by the CA through nervous pathway in diapause adults.