Diapause Induction in Two Geographic Populations of *Cotesia Plutellae* and Its Critical Photoperiodic Response

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Abstract: The critical photoperiod for larval diapause induction was determined in the laboratory for two geographic populations of *Cotesia plutellae* a parasitoid of Diamondback moth (DBM), *Plutellae xylostella* (L.), one population from north China (Changchun) and another from south China (Hangzhou). Also sensitive life stage for diapause induction of *C. plutellae* was studied as well as the influence of the temperature on diapause intensity of both populations. Our study investigated geographical variation in the photoperiodic response for induction of larval diapause at both constant temperature 13 and 15°C. Diapause incidence decreased as the photoperiod increased in both populations although in southern population this criterion of diapause incidence was not found at 15°C. Critical photoperiod was longer at 13°C in northern population than southern population and diapause incidence was higher in northern population than southern population at 13 and 15°C. Also critical photoperiod was longer in southern population than northern one. Our study showed that in *C. plutellae* only first and second larval instar were sensitive to diapause inducing condition. Diapause intensity is highly affected by temperature and latitude. At low temperature and high latitude the diapause intensity was high.

Key words: *Cotesia plutellae*, Diapause, geographic variations, sensitive stage, intensity.

Introduction

Diapause plays a fundamental role in survival during adverse environmental conditions and life cycle synchronization in the majority of insect species in the temperate regions. To survive the winter most temperate insects enter diapause at a fixed development stage, after receiving an environmental cue for diapause induction during certain sensitive stages (Tauber et al., 1986). Photoperiod and temperature are the most important environmental stimuli that influence diapause. Photoperiodic response for diapause induction is an important life history trait for temperate insects in regulating their seasonal life cycle (Tauber et al., 1986). Latitudinal variation in the critical photoperiod for diapause induction occurs because in the temperate zone, temperature and day length show clear latitudinal gradient (Danks, 1994). Most species of insects are exposed to latitudinal gradients of climatic conditions. They may cope with such latitudinal gradients mainly by genetic and partially by phenotypic variations in diapause and related physiological traits (Higashi, 1999).

Diamondback moth (DBM), *Plutellae xylostella* (L.), is the most destructive pest on cruciferous crops worldwide; the annual cost for managing this pest was estimated to be US $ 1 billion in 1992 (Talekar and Shelton, 1993).

C. plutellae has been recommended as an optimal component to design integrated pest management of the diamondback moth, *Plutella xylostella* (L.), in southeastern Asian countries (Talekar and Shelton, 1993). This wasp species has a wide lepidopteron host range and has been registered as a biopesticide. Also it was found to be dominated six parasitoid species reared from DBM larvae and cocoon (Rowell et al 2005).Parasitization by *C. plutellae* produced significant antimetamorphic effect and induced immunodepression of the host, *P. xylostella* (Bae and Kim, 2004).

C. plutellae (Kurdjumov) occupies a wide geographical range， therefore the possibility exists for adaptation of strains to local conditions leading to differences in diapause characteristics between geographically separated populations. Alvi and Momi (1994) examined diapause incidence of two geographic populations of *C. plutellae* at 17°C. They were found that: low latitude population of this wasp did enter diapause, critical day length for diapause induction fell between 12.5 and 13 h at 17°C for the higher latitude population.

Preliminary experiments in the laboratory revealed that the low latitude population of *C. plutellae* could enter diapause. In the present study, latitude, photoperiod and temperature were considered as major factors influencing diapause induction in *C. plutellae*, as reported in many other insects (Kurota and Shimada, 2003). Our study aimed to investigate the following four points: (1) Does the critical photoperiod for diapause induction vary in the two different geographic populations of *C. plutellae* (2) Does the critical photoperiod for diapause induction change according to varying temperature in the two populations of *C. plutellae*? (3) At what developmental stages is *C. plutellae* sensitive to diapause-inducing stimuli? (4) Does the two population of *C. plutellae* vary in their diapause intensity?

Material and methods

Insect cultures and host plants

Two different geographic populations of *C. plutellae* were used in this study. The northern population (NP) originated from 100 cocoons collected in September 2003 on *Brassica* plants at suburbs of Changchun, Jilin province, China (43° 52′ N). The southern population (SP) originated from 100 cocoons collected in November 2003 on *Brassica* plants at suburbs of Hangzhou, Zhejiang province, China (30° 14′ N).

The wasps of the two populations were maintained in the laboratory on a potted cabbage with third and fourth instar larvae of *P. xylostella* as a host. *P. xylostella* was reared on a potted cabbage, *Brassica oleracea* var., capitata, cv. Jingfeng NO 1, according to the method described by Wang et al. (1999). Both the parasitoid and host insect cultures were reared in temperature-controlled rooms (25±1 °C), relative humidity of 60-80% (RH) with 12L: 12D photoperiod. The two parasitoid populations were maintained in separate rearing rooms to ensure isolation.

For producing maternal generation used in the experiments, 30 newly-emerged adult females and 30 males from the culture were transferred and housed in small mating cages for 2 days before being transferred in male-female pairs rearing cages. Rearing cage consists of a clear plastic cup (110 mm in diameter, 110 mm in height) with holes covered with a fine stainless steel mesh on its top and bilateral walls for ventilation and cup lid at the bottom with whole at the center. The plastic cub was placed on top of a 500- ml glass bottle with tap water and the petiole of a cabbage leaf was placed through a whole in the base center of the lid. Cabbage leaf was replaced as needed throughout larval development. Thirty early third larval instar of DBM were placed on a cabbage leaf as host. The exposed host larvae were maintained at 25±1°C LD 12: 12 h until cocoon formation. The cocoons were collected daily and kept in test tubes plugged with cotton balls. The cocoons were maintained in

the same conditions as their immature stages until adult emergence. Emerged adults were kept in the small mating cage under the same previous condition for two days to permit mating prior to the experiment.

Determination of critical day length

The critical day length for diapause induction in two population of *C. plutellae* was examined by allowing the two populations to develop from egg to adults in controlled illuminated chambers under various photoperiods and two different temperature regimes. 30 newly-emerged adult females and 30 males were transferred randomly from the culture and housed in small mating cages for 2 days before using in the experiments. To obtain larvae parasitized by *C. plutellae*, one early third instar larva of DBM was provided to one female in a test tube (18 mm diameter, 80 mm in height), replaced with another one after being stung once by the wasp. Thirty parasitized DBM larvae were placed on a cabbage leaf held in a plastic container. The container was made of a clear plastic cup (110 mm in diameter, 110 mm in height) with holes covered with a fine stainless steel mesh on its top and bilateral walls for ventilation. The cabbage leaf carrying host larvae was fixed to the container base by inserting its petiole through a hole in the base center. The container was placed on top of a 500-ml glass bottle with tap water, and the petiole of the cabbage leaf was kept immersed in water to maintain freshness. The cabbage leaf was replaced when need. Every rearing cage constituted 30 larvae was represented one replicate and five rearing cages were used per each treatment. Rearing cages contained parasitized host larvae by Jilin females were maintained under different photoperiodic regimes: LD 6:18, 8:16, 10:12, 12:12, 14:16,16:8 and 18:6 at constant temperatures of 13 and 15°C. Parasitized host larvae by SP females were placed on a cabbage leaf held in a pre-described rearing cage and maintained under different photoperiodic regimes: LD 6:18, 8:16,10:14, 12:12 and 14:10 h; and LD 2:22, 4:20, 6:18, 8:16,10:14 and 12:12 at constant temperature of 13 and 15°C respectively. The newly spinning cocoons were collected, placed in a test tube (18 × 80mm length) plugged with cotton, and allowed to develop in the same condition as their immature stage. The cocoons, from which no parasitoid had emerged after 26 and 17 day under temperatures of 13 and 15°C, respectively, were dissected under stereomicroscope. The dissected cocoons were classified into two categories: non-diapause (containing dead larva, dead prepupa, pupa or adult) or diapause (containing live larvae or prepupa). The diapause incidence was calculated using the following formula.

% Diapause = (number of cocoons with live larvae or with live prepupa / total cocoon number collected - cocoon number with dead larvae) × 100.

Determination of diapause sensitive life stage of *C. plutellae*

In this experiment, only NP was used. The parasitized host larvae were accomplished by using the same procedure described previously. Collected parasitized host larvae were divided into 75 groups. Each group consisted of 30 larvae caged in a rearing cage with a piece of cabbage leaf. Every five groups represented one treatment as described in fig 1. Rearing cages were then transferred to programmable incubator adjusted at 25°C LD 8:16(non diapause condition).

Depending on the experiment protocol (Fig 2 treatment 1-15), rearing cages were either remained in the original programmable incubator for the remainder of the insect development period (treatment 15) or moved at varying times to another programmable incubator adjusted at 13°C LD 11:13 (diapause-inducing condition) till cocoon formation. Freshly collected cocoons were kept in the same incubator (13°C LD 11:13) as their larval stage (treatments 1, 4, 7) or shifted back to the original

incubator after the time assumed to be necessary to get into pupal stages (treatments 2, 5, 8) or shifted directly after collection to the original incubators (25°C LD 8:16) (treatments 3, 6, 9). In treatment 10 and 11 the egg and the feeding stages remained in the original incubator, and after cocoon formation the newly spinning cocoons were directly moved to another incubator (13° C LD 11:13). Cocoons either remained in the same incubator (13° C LD 11:13) till adult emergence (treatment 11) or were returned back to the original incubator after reaching the pupal stage (treatment 10). Movement of the rearing cages was accomplished according to the duration of the tested stage at that experimental condition.
At the end of each stage treatment, the collected cocoons were dissected under stereomicroscope as described previously, to determine whether they had entered diapause or not.

Diapause intensity

To examine the influence of the temperature on diapause intensity, larvae of both populations were induced to diapause at 13 and 15°C LD 8:16. Cocoons containing diapausing larvae of both NP and SP, were kept under the same conditions as the larval stage until the insects terminated their diapause spontaneously. The number of days taken for the adult to emerge was recorded for each diapausing larva. The period from cocoon formation to adult emergence was defined as diapause intensity in diapausing individuals.
Few individuals from the SP population entered larval diapause under all tested day lengths at 15°C, so we selected the eight hour day length (8 h) under which the diapause proportion were comparatively high to compare the effect of temperature on diapause intensity in the two populations.

Determination of diapausing individuals

The period of development of parasitoid stage inside the cocoon was 26 and 17 days or less at 13°C and 15°C respectively, so individuals whose cocoon stages lasts more than 26 and 17 days at 13°C and 15°C respectively were regarded as diapausing individuals. To firmly confirm that diapause was established, we let the cocoons stay for 20 and 30 days at 15 and 13°C respectively, an additional 3 and 4 days. Day 20 and day 30 after cocoon formation at 13 and 15°C respectively was be selected as day 0.for diapause induction

Statistical analysis

The relationship between diapause incidence (%) and population at each photoperiodic condition was analyzed using a $\chi 2$ test for independence and then the differences in diapause incidence among different photoperiods and between the two populations were analyzed using Turkey-type multiple comparison analysis. The influence of temperature on diapause intensity and the difference between two population in diapause were analyzed by one way analysis of variance and where significant difference were found, means were separated by using student t-test. We analyzed all statistical tests using the SAS software (SAS institute, 2000. v.8.1).

Results

Diapause induction and critical day length

For northern population, diapause incidence increased significantly with decreasing day length under both constant temperatures (13 and 15°C) ($\chi 2$ test, $p<0.05$, Turkey-type multiple comparison test p> 0.05) (Fig 1 a, b). At 13°C almost all individuals of NP entered diapause at a day length of 8, 10, 12 and 14 h, at 16 h, 28.39%, and at 18h, only 16.27% were in diapause (Fig 1a), whereas at 15°C the diapause in NP was 100% only at a day length of 6, 8 and 10 h, at 12 h, 41.61%, at 14h, 30.75%, at 16h, 14.90% and about 8.28% at 18 h were in diapause(Fig I b). Based on these data, we estimated the critical photoperiods (the photoperiod at which 50% of the population enters diapause) for induction of larval diapause in NP to be approximately 15.5 and 11.8 h at 13 and 15°C respectively.

In SP, day length also had significant effect on diapause incidence ($\chi 2$ test, $p<0.05$, Turkey-type multiple comparison test $p> 0.05$). At 13°C a day length of 6, 8 and 10 h induced diapause 62.28, 68.56 and 65.28% respectively (Fig. 1 a). As the day length became longer the diapause incidence was significantly decreased up to 7.14% at a day length of 14 h (Fig. 1 a). At 15°C however, diapause induction was not linked to decreasing day length, although it was significantly affected by the day length ($\chi 2$ test, $p<0.05$). At shorter day lengths of 2 and 4 h, the proportions of individuals in diapause decreased. Again at longer day length 10 and 12 h the diapause occurred at a low level. Shorter (2 and 4 h) and longer (10 and 12 h) day lengths induce same level of diapause in individual larvae, although at 14 h no individuals were in diapause (Fig. 1 b). At day length of 8 and 10h more than half of the individuals averted diapause but was still significantly higher ($\chi 2$ test, $p<0.05$, Turkey-type multiple comparison test $p> 0.05$) than the longer, and shorter, day length. We estimated the critical day length of the SP at 13°C to be 10.5 h., but we could not estimated the critical day length for NP at 15°C since the highest diapause incidence was 35.6% at 8 h day length.

When we compare the critical day length for diapause incidence in the two populations we found that the critical day length for diapause induction in NP was longer than that of SP by about 5 h at 13°C.

The results of 3- way ANOVA indicated that there was geographical variation between the two populations of *C. plutellae* in diapause incidence. Furthermore the diapause incidence was significantly affected by the day length and temperature, and there were significant difference in the interaction of the factors examined (Table 1).

Diapause sensitive life stage of *C. plutellae*

Exposure to diapause inducing conditions during the first or second larval instars to pupal stages or first or second larval instars to prepupal stages or first or second larval instars to the third larval instars resulted in highly significant proportion of diapause. There was no significant difference in diapause induction when we exposed the first or the second larval instar to previous mentioned stages treatments 1 to 6) (Turkey-type multiple comparison analysis**,** $p > 0.05$ Fig. 2) Exposure of the third larval instars separately to diapause inducing conditions or exposure of either the third instar to prepupal or the third larval instars to pupal stages induced diapause in very few individual. (Fig. 2). Exposure of the prepupal stages or the pupal stages to diapause inducing conditions after exposure of all previous stages (egg- all larval stages) to non diapause conditions induce no diapause among the individuals. There was no significant difference in diapause induction when we exposed

the third larval instar or prepupa or pupa to diapause inducing conditions (Turkey-type multiple comparison analysis, $p > 0.05$ Fig. 2).

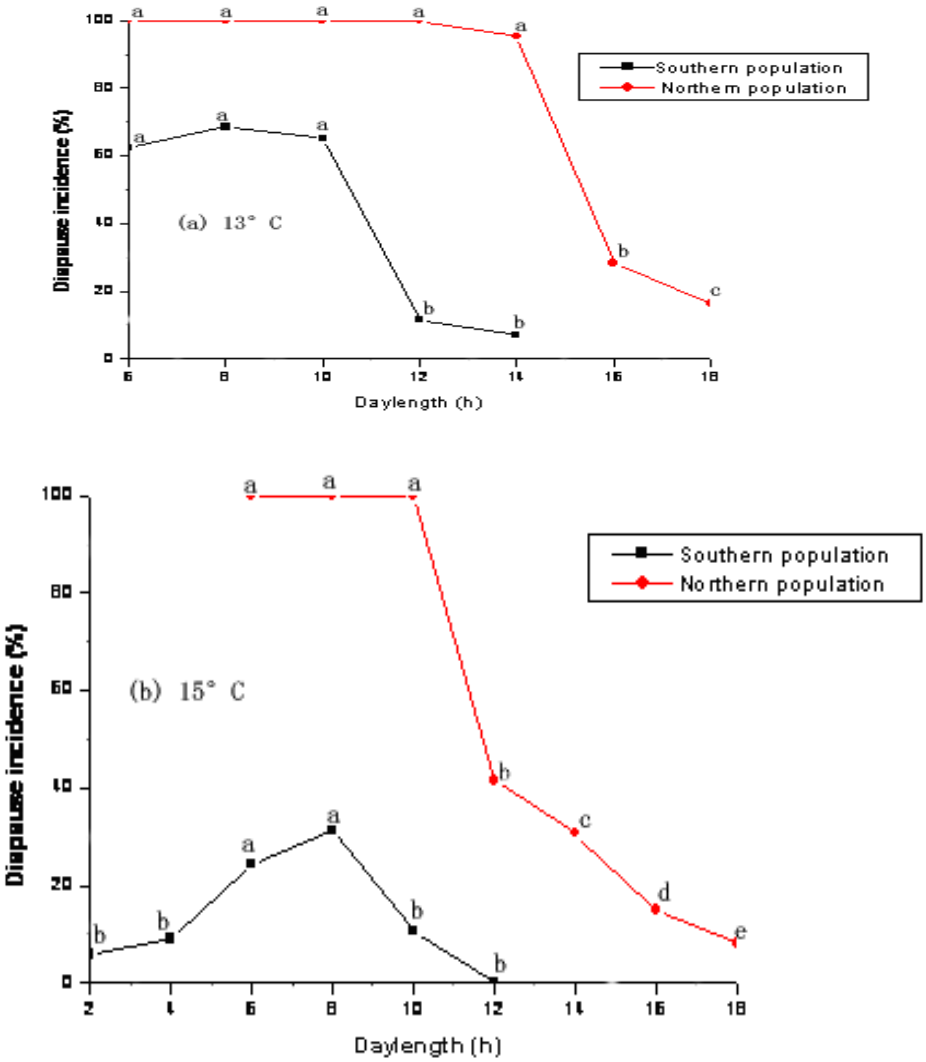

Figure 1. Photoperiodic response curves for larval diapause induction at (a) 13°C and 15°C in *Cotesia plutellae* from Jilin (northern population) and Hangzhou (southern population). Significant differences between the different daylength (h) with in each population are represented by different letters (Tukey-type multiple comparison test).

Table 3 Four-way analysis of variance (ANOVA) testing the effects of geographical location, day length and temperature in diapause incidence of *C. plutellae*.

Effects	DF.	MS	F
Population (A)	1	24979.47	904.13***
Photoperiod (B)	6	6404.17	231.80***
Temperature (C)	5	921.68	33.36***
A×B	1	17884.12	647.32***

A×C	1	516.53	18.70***
B×C	5	356.89	12.92***
A×B×C	4	3909.25	141.50***

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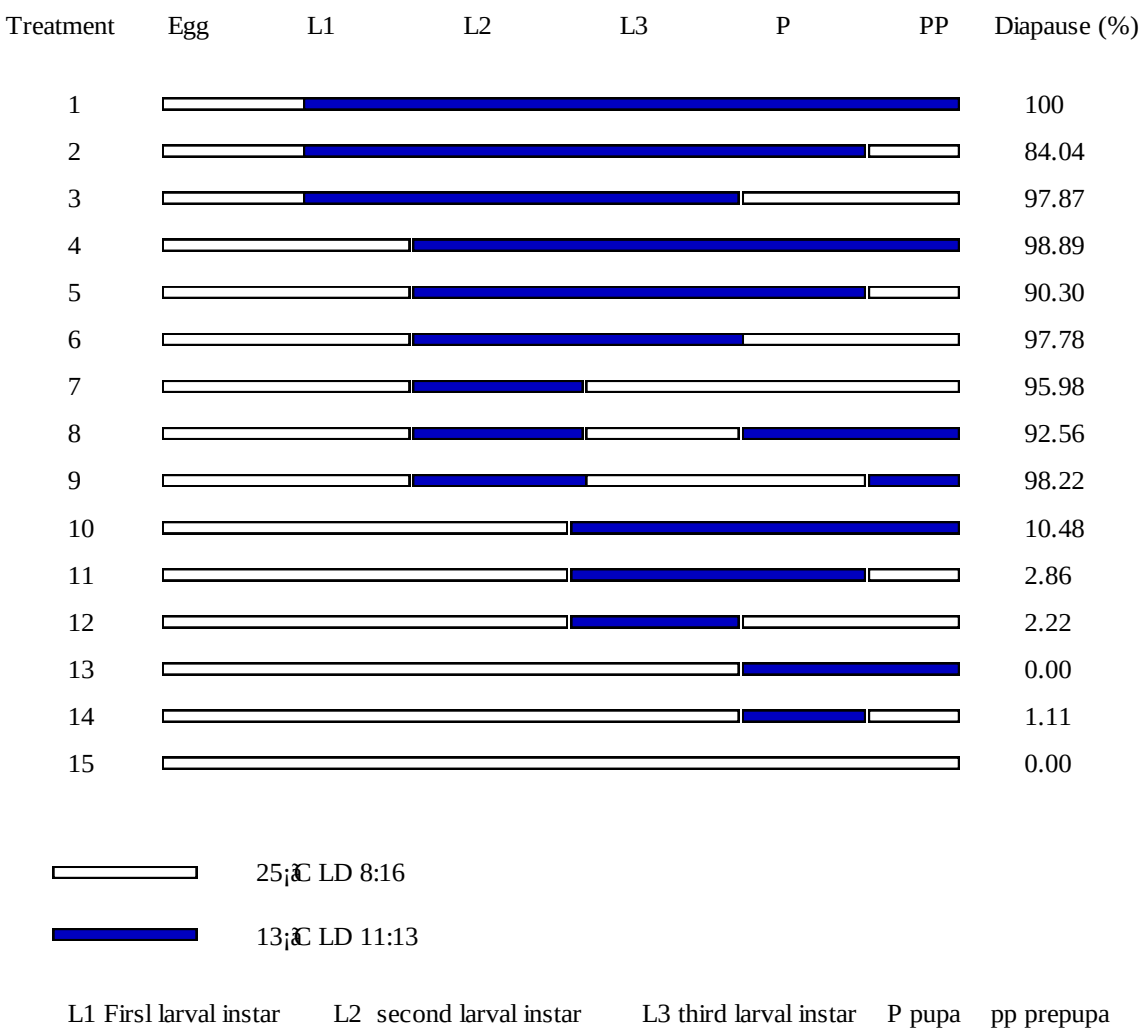

Figure1. Experimental protocols for analysis of the stage life sensitive to diapause-inducing conditions in *Cotesis plutellae*.

Diapause intensity

When the diapause intensity in the two populations was compared under 13 and 15°C LD 8:16 h, mean diapause intensity was significantly longer in NP than SP (two way ANOVA, student t-test Fig 3). Diapause intensity is significantly affected by the temperature (Fig 3). At lower temperature (13°C) the diapause intensity is significantly longer than at relatively higher temperature (15°C) in both populations (Fig 2).

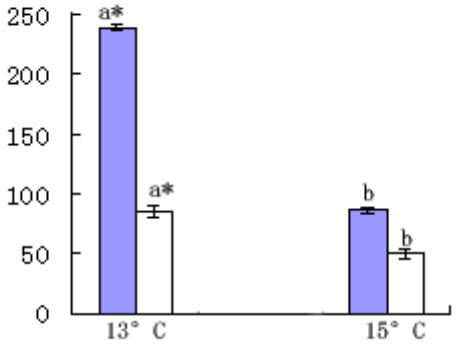

Figure 3 Diapause intensity in two populations of *Cotesia. plutellae* kept under different temperatures and day light of 8h. Means followed by different letters are significantly different (P<0.05, student t test) in the same population. Means followed by asterisks are significantly different between the two populations under the same conditions (P<0.05, student t test). Black column represented northern population (Jilin); White column represented southern population (Hangzhou).

Discussion

Critical day length

In the present study there are distinct differences in the incidence of diapause between the two populations even under the same conditions (Fig 1 a, b). Diapause incidence under both constant temperatures 13°C and 15°C and all day light length was more in population from higher latitude than from lower latitude population. The critical day length for diapause induction was longer in the NP than in SP of *C. plutella.* Thus, selection for diapause response in the North is expected to be stronger than in the South resulting in a higher proportion of diapausing individuals. A similar latitudinal cline has been previously described in other species (Scharf *et al*., 2010; Leisnham *et al*., 2011). For example, in *D. melanogaster*, the incidence of adult diapause was positively correlated with latitude in populations in Eastern North America (Schmidt *et al*., 2005). At more northern latitudes, when the favorable season becomes shorter, winter arrives earlier and the organisms enter diapause earlier in the year when days are longer to prevent loss of insect developmental stages that can not stand the harsh winter conditions (Tauber et al., 1986). Among a wide variety of arthropods, the critical day length cuing seasonal activity increases regularly with increasing latitude or altitude, thereby providing an apparent adaptive photoperiodic response to the latitudinal gradient in season length (Tayler and Spalding, 1986). Bradshaw et al. (2004) defined adaptive photoperiodic changes as genetic changes in photoperiodic response that conform to the seasonal environmental changes and can be shown to improve fitness in those environments.

In NP the diapause incidence was high at shorter day length and it declined as the day length became longer. The critical day length for diapause induction was longer at 13°C (15.5 h) than at 15°C (11.5 h). Other studies have investigated photoperiod effect (Leisham et al. 2011, Yee et al. 2012), which can provide information on how photoperiod as an independent cue may alter life history and behavior. Temperature is another less reliable factor that plays a modifying role in the photoperiodic response of many insects. Our results confirm the importance of temperature on the photoperiodic response of *C. plutellae*, where a longer critical day length occurs at 13°C versus 15°C.

In SP the highest diapause percentage was found at 6, 8 and 10 h day length at 13°C. The diapause incidence decreased as the photoperiod increased. At 15°C the diapause incidence decreased both at the extremely shorter and longer photoperiods. The highest diapause incidence was found at 6 and 8 h day light, whereas the shorter and longer photoperiods effectively prevented diapause. In many long-day insects, diapause induction declined with extreme long nights (>19 h). This behavior was shown by many other insect, such as the cabbage butterfly, *Pieris brassicae*; Indian meal moth, *Plodia interpunctella* (Masaki and Kikukawa, 1981); the linden bug, *Pyrrhocoris apterus* (Saunders 1983); the fly, *Chymomyza costata* (Yoshida and Kimura, 1995); the spider mite, *Tetranychus urticae* (Kroon et al., 1997) and cabbage beetle, *Colaphellus bowring* (Wang et al., 2004). In SP, Less than 50% of the individuals entered diapause at 15°C under all tested day length conditions. *C. plutellae* from lower latitude had no quick sense to enter diapause and this was clear from the diapause proportion under all photoperiodic conditions at 15°C. In contrast Alvi and Momi (1994) reported that *C. plutellae* population from lower latitude does not enter diapause. The inconsistent of Alvi and Momi results and our results related to the parental generation conditions. Alvi and Momi reared the parental generation under long day condition (16 h), whereas in this present work the parental generation was reared under short day conditions (12 h). In *C. plutellae* diapause incidence in progeny generation was highly affected by the photoperiodic condition experienced by the parental generation (Unpublished data). Furthermore SP was obtained from a city located in subtropical zone, and so the adaptive significance of diapause for the SP may not entirely correspond to that for the temperate population.

Sensitive life stage for diapause induction in *C. plutellae*

The present study indicates that both first and second instar larvae of *C. plutellae* are sensitive to diapause-inducing stimuli. There is no significant difference in response to diapause inducing stimuli between the first instar (which experienced the cumulative effects of diapause inducing-conditions throughout the whole larval development) and second instar (Fig.2). Alvi and Momi, (1994) reported that the sensitive developmental stage for diapause inducing stimuli was in the period of second and third larval instar. Our results are consistent with their result since we found that second larval instar was sensitive to diapause inducing stimuli. Again Alvi and Momi, 1994 stated that the first larval instars are not sensitive to diapause inducing condition. This observation is inconsistent with our results. Alvi and Momi, (1994) in their experiment they exposed the first larval instar to diapause inducing conditions then shifted to the non diapause conditions when it reached the third larval instar. The decision to diapause should not be viewed as a simple “yes or no” decision made at a single moment in time, but rather it is the culmination of a series of events. In flesh fly the diapause program can be averted at many points along the way, right up to the time that diapause is normally manifested. Diapause program can be aborted at the last minute, and the attempt to induce diapause by offering the correct signals only during the later phases of development will not be sufficient to induce diapause (reviewed by Denlinger et al., 2005). Our result agrees with these statements since we kept the first larval instar under diapause inducing conditions till the third larval instar (the diapausing stage). Our results also are consistent with those reported for other insect species, the dagger moth, *Acronicta rumicis* L., the bollworm, *Helicoverpa zea* (Boddie), and the tobacco budworm, *Helicoverpa* (5 *Heliothis*) *virescens* (Fabricius), in which sensitivity to photoperiod has been shown to appeared in all larval stages preceding the diapausing pupal stage (Goryshin and Tyshchenko,1970). Third (last) larval instar, prepupa and pupal stages (cocoon stage) had no sensitivity to diapause inducing conditions. This may be due to the physiological (lack of photosensitivity) rather than ecological status, since they were subjected to the same conditions as the first and second instar

larvae. This observation is consistent with the results of Alvi and Momi, (1994). We did not test the sensitivity of the egg stages separately from the larvae. Photoperiodic sensitivity in eggs has not been reported in studies of diapause induction involving other insect species (Goryshin and Tyshchenko, 1970).

Diapause intensity

In the present study, the intensity of diapause induced under the two experimental combinations examined in both populations was conspicuously different, although the post-feeding stage in diapause larvae consists of the periods of diapause and postdiapause morphogenesis. Differences in the intensity of diapause are often found among geographical populations (Masaki, 2002; Tobin et al., 2002). When assessed on the basis of the time course for adult emergence from diapausing cocoons, diapause is significantly more intense at lower temperature (13°C) than at relatively higher temperature (15°C). These observations are consistent with the results of Tachibana and Numata (2004) who showed that diapause intensity is influenced by the photoperiod and temperature experienced by the larvae themselves. Further more NP had significantly intense diapause than SP. The major role of winter diapause may be to prevent the restart of development before the depth of winter since NP was obtained from a city with a longer winter season than the city from which SP was obtained.

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