

Effects of thermo-photoperiod on induction and termination of hibernation in *Chilo partellus* (Swinhoe)

M. K. Dhillon*, F. Hasan, A. K. Tanwar and
 A. S. Bhadauriya

Division of Entomology, ICAR-Indian Agricultural Research Institute,
 New Delhi 110012, India

Abstract

Determination of critical threshold for induction and termination of diapause (hibernation) are important for better understanding the bio-ecology and population dynamics of *Chilo partellus* (Swinhoe) under varying climatic conditions. We studied initiation and termination of hibernation under five temperature and photoperiod regimes viz., 27°C + 12L:12D, 22°C + 11.5L:12.5D, 18°C + 11L:13D, 14°C + 10.5L:13.5D and 10°C + 10L:14D under fixed and ramping treatments, and the observations were recorded on various phenological and developmental characteristics at weekly intervals. Present studies revealed that the induction of hibernation in *C. partellus* larvae takes from 46 to 56 days depending upon temperature and photoperiod conditions. Induction of hibernation varied from 7.9 to 18.3% across treatment conditions, indicating that not all *C. partellus* larvae undergo diapause under prevailing environmental conditions. Weight, length and head capsule width of diapausing larvae were found significantly lower than the non-diapausing larvae. The non-diapausing *C. partellus* larvae required a thermal threshold of 1068 degree-days under ambient conditions, while in case of hibernating larvae it varied significantly across treatment conditions. Diapausing larvae underwent up to five supernumerary moults, wherein highest percentage of diapausing larvae (35.7%) exhibited two supernumerary moults. The developmental time of diapausing larvae varied from 94.9 to 160.4 days across treatments. A population loss of 17.2–28.3% was recorded in *C. partellus* due to hibernation, which has implications for population buildup of post-hibernation first brood and management strategies.

Keywords: Abiotic factors, *Chilo partellus*, degree-days, diapause induction, diapause termination, hibernation

(Accepted 1 September 2016; First published online 10 November 2016)

Introduction

Diapause in insects has evolved as an important life history component at a particular stage, which plays a key role in controlling ecology, phenology, and physiology (Andrewartha, 1952; Xiao *et al.*, 2010). Diapause is genetically programmed physiological state of arrested metabolic activity that enables an insect to survive under predictable adverse climatic

conditions and diminishing resources needed for development, reproduction and mutagenesis (Neal *et al.*, 1997; Youcum *et al.*, 2011; Lehmann *et al.*, 2015), and synchronizes activity cycles with favorable conditions thus ensuring effective resource utilization (Tauber *et al.*, 1986; Arbab, 2014). During diapause, the insect passes through several processes such as induction, maintenance, termination and post-diapause development (Xiao *et al.*, 2010; Hodek, 2012), and undergoes several changes such as suppression of developmental and reproductive functions, accumulation of metabolic reserves, and reduced metabolic activity (Beck, 1980; Qiang *et al.*, 2012). Diapause is not always an adaptive advantage as it can also pose a threat to insects, for example, if an insect

*Author for correspondence
 Phone/Fax: +91-11-25842482
 Email: mukeshdhillon@rediffmail.com

enter diapause early in the season or delay in emergence from diapause lead to copulation disruption and laying of unfertilized eggs, whereas delayed entrance or early termination of diapause might expose insects to harsh climatic conditions, which ultimately kill them (Irwin & Lee, 2000; Jiang *et al.*, 2010; Arbab, 2014). Furthermore, exposure of hibernating or overwintering individuals to further low temperature contribute to depletion of energy reserves, which directly affect survival, post-diapause development, reproduction, fecundity, and adult longevity (Ellers & van Alphen, 2002; Xiao *et al.*, 2010).

Spotted stem borer, *Chilo partellus* (Swinhoe) is one of the most serious constraints in increasing yield potential of maize and sorghum, causing yield loss of about 18–25% under different agro-climatic conditions in Asia and Africa (Khadioli *et al.*, 2014; Dhaliwal *et al.*, 2015). *Chilo partellus* undergoes facultative diapause as mature larvae inside the old stems or stubbles, and is an important component of its biology, population dynamics and geographic distribution (Ofomata *et al.*, 1999; Kfir *et al.*, 2002). During diapause, larvae of *C. partellus* moult several times designated as supernumerary moults (Scheltes, 1978). Occurrence of supernumerary moults and consumption of food during diapause have also been reported in several other stem borer species such as *Diatraea saccharalis* (Fab.) (Roe *et al.*, 1984), *Chilo suppressalis* Walker (Koidsumi & Makino, 1958; Xiao *et al.*, 2010; Qiang *et al.*, 2012), and *Sesamia nonagrioides* (Lefebvre) (Gadenne *et al.*, 1997). Diapause duration i.e., from its induction to termination has also been extensively studied in *Busseola fusca* (Fuller) (Kfir, 1993). The genetic determination of locally adapted life-history traits such as diapause and polyphenism in insects are important to understand their bio-ecology and population dynamics in response to climate change and the host plants on which it feeds (Sotherlind & Nylin, 2011).

Initiation and termination of diapause is genetically pre-defined phenomenon, triggered by a number of stimuli such as temperature, photoperiod, humidity and food availability (Hodek, 2012; Fischer *et al.*, 2014), and controlled by neuro-hormones (Nijhout, 1975). *Chilo partellus* undergoes hibernation (winter diapause) under North Indian conditions. The information on critical threshold conditions for induction and termination of winter diapause, phenology of diapausing larvae, supernumerary moults, insect population loss due to diapause, etc. are some of the important points, which needs greater attention for better understanding the bio-ecology and population dynamics of insect pests for sustainable crop protection. Therefore, present studies were undertaken to examine: (i) effects of fluctuating and constant temperature coupled photoperiod treatments on the induction and duration of winter diapause, and phenology of diapausing and non-diapausing individuals; (ii) degree-day accumulations for the development of diapausing and non-diapausing individuals; (iii) occurrence of supernumerary moults during the course of hibernation, and (iv) population loss due to winter diapause in *C. partellus*. Such information will be highly useful for designing novel management strategies.

Materials and methods

Insect rearing and experimental conditions

Spotted stem borer, *C. partellus* larvae were collected from experimental field of Division of Entomology, ICAR-Indian

Agricultural Research Institute (Latitude – 28°38'23" N and Longitude – 77°09'27"E, height above mean sea level is 228.61 m), New Delhi, India and reared on artificial diet (Sharma *et al.*, 1992) at 27 ± 1°C, 70 ± 5% relative humidity, and 12 h photoperiod under laboratory conditions. Adults emerged from the culture were released in oviposition cages. The oviposition cages were covered with wax-paper from outside to serve as oviposition substrate. The wax-papers were changed daily, and the papers with eggs were kept at 27 ± 1°C for hatching and use in different experiments. Relative humidity was 70 ± 5% during all the experiments. To determine the critical climatic conditions that enable *C. partellus* larvae to enter diapause, five temperature regimes i.e., 27 ± 1°C, 22 ± 1°C, 18 ± 1°C, 14 ± 1°C and 10 ± 1°C with respective photoperiod combinations 12:12, 11.5:12.5, 11:13, 10.5:13.5 and 10:14 (L:D), were calibrated in separate incubators (Calton, Narang Instruments Pvt. Ltd., India). These temperature and photoperiod combinations were framed in order to provide an environment, which mimics the natural conditions and induce hibernation in the test insect.

Diapause determination

Three basic criteria *viz.*, behavioral, morphological and physiological were taken into account to determine larval diapause, and differentiate diapausing and non-diapausing larvae. Under the behavioural criteria it was noted that diapausing larvae fail to pupate (Scheltes, 1978), construct a resting site (diapause chamber), and exceeded the normal development time. The morphological characteristics *viz.*, absence of cuticular pigmentation (Scheltes, 1978), absence of asetose tubercles (Mathez, 1972), turning of larval body from creamy to milky white, and turning of prothoracic shield from light brown to creamy in colour were determined for the diagnostics of larval diapause. Third criteria i.e., physiological changes that enable diapause larvae to moult extra (supernumerary moult) exceeding their genetically fixed ecdysis (normally six instars) (Kfir, 1991) was also taken into consideration to ensure larval diapause.

Development, survival and induction of diapause in C. partellus at different temperature and photoperiod ramping regimes

This experiment was set up with 50 neonate *C. partellus* larvae released on artificial diet in plastic jars (1000 ml capacity) per replication and there were three replications in a completely randomized design. Since, the neonate larvae of *C. partellus* exhibit strong phototactic behavior, the experimental jars were wrapped with a black paper immediately after larval inoculation, leaving only the diet portion uncovered to allow the larvae to properly settle into diet in the culture room at 27 ± 1°C and 12L: 12D photoperiod. Three days after inoculation, the black paper sheets were removed and the larvae shifted to calibrated experimental conditions, i.e., 27 ± 1°C and 12L: 12D photoperiod in the incubator. One week after inoculation, five randomly selected individual larvae per replication were removed from the experimental jars for observations on larval weight, head capsule width and larval length, and averaged thus making three replications. After observations the larvae were placed back to respective jars. Observations on larval weight were recorded using Precision electronic balance (CB-Series, Contech); and the head capsule width and larval length were recorded using Leica StereoZoom microscope

(Leica Microsystems Ltd, Heerbrugg, Switzerland). After completion of observations, the experimental jars were shifted to $22 \pm 1^\circ\text{C}$ and 11.5L:12.5D in the incubator. After 1 week of exposure to each temperature and photoperiod conditions, similar morphometric observations as mentioned above were also recorded under each experimental condition, and the experimental jars were shifted to further lower temperature and photoperiod regimes, i.e., $18 \pm 1^\circ\text{C} + 11\text{L}:13\text{D}$, $14 \pm 1^\circ\text{C} + 10.5\text{L}:13.5\text{D}$, and $10 \pm 1^\circ\text{C} + 10\text{L}:14\text{D}$ at weekly intervals. Weekly exposure of the test insects to $27 \pm 1^\circ\text{C} + 12\text{L}:12\text{D}$, $22 \pm 1^\circ\text{C} + 11.5\text{L}:12.5\text{D}$, $18 \pm 1^\circ\text{C} + 11\text{L}:13\text{D}$, $14 \pm 1^\circ\text{C} + 10.5\text{L}:13.5\text{D}$, and $10 \pm 1^\circ\text{C} + 10\text{L}:14\text{D}$ conditions was designated as ramping 1. After completion of ramping 1, the test insects were exposed back from lower to higher temperature and photoperiod regimes at weekly intervals and designated as ramping 2. The rampings 3 and 4 followed the same trend as rampings 1 and 2, respectively. Similar observations on larval weight, head capsule width, and larval length were continued during rampings 3 and 4 till all the test individuals completed larval development. During the exposure of test *C. partellus* larvae to different temperature and photoperiod regimes during each ramping, the diapausing larvae (~45 days after diapause) were separated and reared on fresh diet at $27 \pm 1^\circ\text{C}$, 12L:12D and 70 ± 5% RH, and the observations were recorded and expressed as percent larval survival, larval duration (days), and population loss due to diapause.

Development, survival and induction of diapause in C. partellus at constant temperature and photoperiod regimes

The experiment on induction of diapause in *C. partellus* at constant temperature and photoperiod regimes was designed at five temperature and photoperiod regimes, i.e., $27 \pm 1^\circ\text{C} + 12\text{L}:12\text{D}$, $22 \pm 1^\circ\text{C} + 11.5\text{L}:12.5\text{D}$, $18 \pm 1^\circ\text{C} + 11\text{L}:13\text{D}$, $14 \pm 1^\circ\text{C} + 10.5\text{L}:13.5\text{D}$, and $10 \pm 1^\circ\text{C} + 10\text{L}:14\text{D}$ in separate incubators. Fifty neonate *C. partellus* larvae were released on artificial diet in plastic jars (1000 ml capacity) per replication and there were three replications for each set of temperature and photoperiod regimes in a completely randomized design. Similar to previous experiment, the experimental jars inoculated with neonate *C. partellus* larvae were kept wrapped with a black paper sheet for 3 days to disrupt phototactic behavior and settle on the artificial diet. Keeping in view the slow rate of larval development at lower temperature regimes and length of larval duration at optimum temperature, the observations were started after 3 weeks of exposure to each treatment condition, and repeated at 5 day intervals till pupation. The observations were recorded on larval morphometrics, larval and pupal developmental durations, larval survival and adult emergence. The observations on larval morphometrics, i.e., larval weight, head capsule width, and body length were recorded on randomly selected five larvae per replication. The larvae were placed back in the respective jars after observations. The *C. partellus* larvae displaying diapause symptoms under each of the experimental conditions were removed from their respective treatment regimes, and reared separately under similar treatment conditions. After 45 days of entering diapause the larvae were provided with fresh artificial diet. The diapausing *C. partellus* larvae from each treatment regime were collected and counted separately, and observations were recorded and expressed as percent larval survival, larval duration (days), and percent population loss due to diapause.

Determination of supernumerary moults

There is little knowledge available on number of additional ecdysis or supernumerary moults the diapausing *C. partellus* larvae undergoes, and therefore, we performed an additional experiment to determine the actual number of supernumerary moults that occur during diapause in this insect. A total of 30 diapausing *C. partellus* larvae of same age were collected singly in the 6-well tissue culture plates with a piece of artificial diet, and placed at $27 \pm 1^\circ\text{C} + 12\text{L}:12\text{D}$ in the incubator. Daily observations were made on larval–larval ecdysis in each of the test insect and continued till pupation. The diet when became hard and dry was replaced with fresh diet. The dead larvae observed during the experiments were removed and excluded from data analysis. The data were expressed as distribution of percentage of diapausing larvae entered pupation according to number of supernumerary moults.

Statistical analysis

The percentage data were subjected to Arcsine transformation, and the analysis of variance (ANOVA) were carried out using statistical software SAS[®] version 9.2. Before analysis all the data sets were checked for normality using Kolmogorov–Smirnov (K–S) test. The data on larval morphometric parameters (weight, length, and head capsule width), developmental durations and thermal requirements (degree-days) in diapausing and non-diapausing larvae across temperature and photoperiod rampings were subjected to repeated measurements one-way ANOVA, considering weekly intervals data in particular ramping as a factor, and the significance of differences were judged by χ^2 test. Since, there were no symptoms of diapause induction in *C. partellus* during temperature and photoperiod rampings 1 and 2, and larval development was stopped after certain period of exposure at constant temperature and photoperiod treatments *viz.*, $18 \pm 1^\circ\text{C} + 11\text{L}:13\text{D}$, $14 \pm 1^\circ\text{C} + 10.5\text{L}:13.5\text{D}$ and $10 \pm 1^\circ\text{C} + 10\text{L}:14\text{D}$, thus the data from these treatments were not included in further analysis. The differences between physiological states (diapausing and non-diapausing) across temperature and photoperiod rampings (rampings 3 and 4) for larval morphometric parameters (weight, length, and head capsule width), larval duration, pupal duration, and thermal requirements (degree-days) were compared using repeated measurements two-way ANOVA, and the significance of differences were judged by χ^2 test. The data on different biological parameters (larval duration, larval survival, pupal period, pupal mortality, and adult emergence), and diapause initiation and termination parameters (diapause induction, diapaused larval survival, and *C. partellus* population loss due to diapause) under constant temperature and photoperiod regimes were analyzed using one-way ANOVA, while larval morphometric parameters (weight, length, and head capsule width), developmental durations and thermal requirements (degree-days) of diapausing and non-diapausing larvae and their interactions with constant temperature and photoperiod regimes were analyzed using two-factor factorial analysis, and the significance of differences were checked by F-test and treatment means were compared using least significant differences (LSD) at $P \leq 0.05$.

Percent population loss due to diapause was calculated using the formula: Percent population loss due to diapause = (number of larvae died during diapause/total number of larvae entered diapause) × 100.

Table 1. Effect of different temperature and photoperiod rampings on the morphometric parameters of non-diapausing and diapausing *Chilo partellus* larvae.

Temperature and photoperiod rampings	Non-diapausing larvae			Diapausing larvae		
	Larval weight (mg larva ⁻¹)	Larval length (mm)	Head capsule width (mm)	Larval weight (mg larva ⁻¹)	Larval length (mm)	Head capsule width (mm)
Ramping 1	3.2 ± 0.03d	3.8 ± 0.06d	0.5 ± 0.00d	ND	ND	ND
Ramping 2	36.6 ± 0.50c	9.1 ± 0.19c	0.9 ± 0.01c	ND	ND	ND
Ramping 3	55.8 ± 1.60b	9.6 ± 0.09b	1.0 ± 0.01b	53.5 ± 0.76a	9.1 ± 0.38a	1.0 ± 0.00a
Ramping 4	114.8 ± 1.20a	11.2 ± 0.18a	1.2 ± 0.01a	56.2 ± 0.88a	8.9 ± 0.2a	1.0 ± 0.00a

Values within column followed by different letters are significant at $P=0.05$. ND=no diapause recorded during rampings 1 and 2. NS= nonsignificant at $P=0.05$. $N=15$ in every ramping for each type of larvae.

Table 2. Larval and pupal durations and numbers of degree-days required for the development of non-diapausing and diapausing populations of *Chilo partellus* across different temperature and photoperiod rampings.

Physiological state	Larval duration (days)	Pupal duration (days)	Thermal requirement for larval development (degree-days)
Non-diapause	108.0 ± 0.08b	12.7 ± 0.84a	1731.1 ± 7.9b
Diapause	160.4 ± 0.79a	15.9 ± 0.46a	3139.3 ± 16.6a

Values within column followed by different letters are significant at $P=0.05$. NS= nonsignificant at $P=0.05$.

The degree-days were calculated using a modified formula given by Dhillon & Sharma (2007): total degree-days = number of exposure days to particular temperature × respective temperature till completion of postembryonic development (e.g., total degree-days = $d_1 \times t_1 + d_2 \times t_2 + d_3 \times t_3 + \dots + d_n \times t_n$; where d = days of exposure; t = particular temperature).

Results

Effect of different temperature and photoperiod ramping regimes on morphometric and developmental parameters of diapausing and non-diapausing larvae of *C. partellus*

The larval weight, body length, and head capsule width of non-diapausing *C. partellus* larvae across different temperature and photoperiod ramping regimes varied from 3.2 to 114.8 mg larva⁻¹, 3.8–11.2 mm, and 0.5–1.2 mm, respectively (table 1). There was significant influence of temperature and photoperiod ramping regimes on the weight ($\chi^2 < 0.001$), body length ($\chi^2 < 0.001$), and head capsule width ($\chi^2 < 0.001$) of non-diapausing *C. partellus* larvae. The larval weight, body length, and head capsule width of non-diapausing *C. partellus* larvae were significantly higher at ramping 4 as compared with other ramping regimes (table 1). There were no symptoms of diapause induction in *C. partellus* exposed to different temperature and photoperiod regimes during rampings 1 and 2, while at rampings 3 and 4 the *C. partellus* larvae started entering diapause. The differences for larval weight ($\chi^2 = 0.14$), body length ($\chi^2 = 0.61$), and head capsule width ($\chi^2 = 0.39$) of diapausing *C. partellus* larvae during ramping 3 and 4 were statistically nonsignificant (table 1). However, the differences for these traits between diapausing

and non-diapausing *C. partellus* larvae, during rampings 3 and 4, and their interactions were significant ($\chi^2 < 0.001$).

The diapause larvae of *C. partellus* took significantly longer time to turn into pupa as compared with non-diapausing counterpart ($\chi^2 < 0.001$) during different temperature and photoperiod rampings (table 2). Duration of pupal eclosion during different temperature and photoperiod rampings in diapause population was comparatively longer than the non-diapausing population, however, the differences were nonsignificant ($\chi^2 = 0.102$). Thermal requirements for overall development of *C. partellus* larvae across different temperature and photoperiod rampings was significantly more for diapausing ($\chi^2 < 0.001$) than the non-diapausing larvae (table 2).

Effect of different constant temperature and photoperiod regimes on morphometric and developmental parameters of diapausing and non-diapausing larvae of *C. partellus*

The neonate *C. partellus* larvae exposed to constant temperatures and photoperiods viz., 18 ± 1°C + 11L:13D, 14 ± 1°C + 10.5L:13.5D, and 10 ± 1°C + 10L:14D survived upto 105.0, 104.5, and 91.0 days, respectively and failed to pupate, however at 27 ± 1°C + 12L:12D and 22 ± 1°C + 11.5L:12.5D the insect successfully completed the development. Furthermore, the larval duration of *C. partellus* exposed to 27 ± 1°C + 12L:12D was significantly shorter ($F = 1683.46$; $df = 4,8$; $P \leq 0.001$) than those exposed to 22 ± 1°C + 11.5L:12.5D (table 3). However, the larval survival at 27 ± 1°C + 12L:12D and 22 ± 1°C + 11.5L:12.5D was on par with each other ($F = 1.58$; $df = 1,2$; $P = 0.330$). The pupal period was significantly shorter ($F = 57.7$; $df = 1,2$; $P = 0.017$) and pupal mortality lower ($F = 49.0$; $df = 1,2$; $P = 0.020$) at 27 ± 1°C + 12L:12D than at 22 ± 1°C + 11.5L:12.5D. The adult emergence was significantly higher at 27 ± 1°C + 12L:12D ($F = 16.74$; $df = 1,2$; $P = 0.040$) than at 22 ± 1°C + 11.5L:12.5D (table 3).

A comparison between diapausing and non-diapausing *C. partellus* larvae at constant temperature and photoperiod treatments viz., 27 ± 1°C + 12L:12D and 22 ± 1°C + 11.5L:12.5D for various morphometric parameters such as larval weight, larval length, and larval head capsule width, was made using two-way ANOVA. The two-way ANOVA suggested significant differences for weight ($F = 1183.24$, $df = 1,2$, $P \leq 0.001$), length ($F = 17.73$, $df = 1,2$, $P = 0.006$), and head capsule width ($F = 6.25$, $df = 1,2$, $P = 0.046$) of diapausing and non-diapausing larvae across thermo-photoperiod treatments. The diapausing *C. partellus* larvae had significantly lower larval weight, shorten length, and head capsule width than the non-diapausing larvae at both 27 ± 1°C + 12L:12D and 22 ± 1°C + 11.5L:12.5D, respectively (table 4).

Table 3. Development and survival of *Chilo partellus* at different constant temperature and photoperiod regimes.

Temperature (°C) and photoperiod (L:D) treatments	Larval duration (days)	Larval survival (%)	Pupal period (days)	Pupal mortality (%)	Adult emergence (%)
27 (12:12)	39.5 ± 0.87b	67.3 ± 1.33a	9.8 ± 0.16b	20.0 ± 0.57b	47.3 ± 0.88a
22 (11.5:12.5)	66.5 ± 0.66a	62.7 ± 2.40a	11.9 ± 0.18a	29.4 ± 0.88a	33.3 ± 1.85b
18 (11:13)	105.0	NDR	NDR	NDR	NDR
14 (10.5:13.5)	104.5	NDR	NDR	NDR	NDR
10 (10:14)	91.0	NDR	NDR	NDR	NDR
LSD ($P = 0.05$)	2.22	NS	1.63	2.22	8.88

Values within column followed by different letters are significant at $P = 0.05$. NDR = no development recorded after particular duration, and data were not included in further analysis. NS = nonsignificant at $P = 0.05$

Table 4. Effects of constant temperature and photoperiod on the morphometric parameters of non-diapausing and diapausing *Chilo partellus* larvae.

Temperature (°C) and photoperiod (L:D) treatments	Weight (mg)		Length (mm)		Head capsule width (mg)	
	Non-diapause	Diapause	Non-diapause	Diapause	Non-diapause	Diapause
27 (12:12)	134.55 ± 3.14Aa	88.33 ± 0.5Ab	16.15 ± 0.14Aa	14.05 ± 0.15Ab	1.53 ± 0.00Aa	1.41 ± 0.00Ab
22 (11.5:12.5)	86.26 ± 0.89Ba	83.58 ± 0.1Bb	15.93 ± 0.19Ba	13.22 ± 0.09Bb	1.49 ± 0.02Ba	1.32 ± 0.01Bb
For comparing	LSD ($P = 0.05$)		LSD ($P = 0.05$)		LSD ($P = 0.05$)	
Treatments	1.54		0.179		0.021	
State	1.54		0.179		0.021	
Treatment × state	2.19		0.253		0.029	

Values within column and rows for each parameter followed by different capital and small letters, respectively are significant at $P = 0.05$.

Similarly, the developmental parameters i.e., larval duration, pupal duration, and thermal requirements (degree-days) were also compared between diapausing and non-diapausing *C. partellus* larvae at constant temperature and photoperiod treatments *viz.*, $27 \pm 1^\circ\text{C} + 12\text{L}:12\text{D}$ and $22 \pm 1^\circ\text{C} + 11.5\text{L}:12.5\text{D}$. Present studies revealed that the non-diapausing *C. partellus* larvae under ambient conditions ($27 \pm 1^\circ\text{C} + 12\text{L}:12\text{D}$) require a thermal threshold of 1068 degree-days. The two-way ANOVA showed significant differences for larval duration ($F = 9.41$, $df = 1,2$, $P = 0.022$), pupal duration ($F = 8.19$, $df = 1,2$, $P = 0.029$), and thermal requirement (degree-days: $F = 12.03$, $df = 1,2$, $P = 0.013$) of diapausing and non-diapausing larvae across thermo-photoperiod treatments. The diapausing *C. partellus* larvae had significantly longer larval and pupal durations, and higher thermal requirements than the non-diapausing larvae at both $27 \pm 1^\circ\text{C} + 12\text{L}:12\text{D}$ and $22 \pm 1^\circ\text{C} + 11.5\text{L}:12.5\text{D}$, respectively (table 5).

Initiation and termination of diapause

There was significant variability in induction of diapause ($F = 11.87$, $df = 2,4$, $P = 0.021$), survival of diapaused larvae ($F = 28.07$, $df = 2,4$, $P = 0.004$), and *C. partellus* population loss due to diapause ($F = 28.10$, $df = 2,4$, $P = 0.004$) across different temperature and photoperiod treatments (table 6). The observations on diapause during different temperature and photoperiod rampings revealed that the numbers of diapaused larvae recovered were significantly higher during ramping 4 (12.9%) than ramping 3 (5.5%) ($F = 38.57$, $df = 1,2$, $P = 0.02$). However, there were no significant differences between ramping 3 and 4 for survival of diapaused larvae ($F = 0.91$; $df = 1,2$; $P = 0.64$) and *C. partellus* population loss due to diapause ($F = 0.92$, $df = 1,2$, $P = 0.43$). No significant differences were observed in induction of diapause under $22 \pm 1^\circ\text{C} + 11.5\text{L}:12.5\text{D}$

(14.99%) and those exposed to different temperature and photoperiod ramping (18.3%) conditions, being significantly lower at $27 \pm 1^\circ\text{C} + 12\text{L}:12\text{D}$ (table 6). The population loss due to diapause under constant $22 \pm 1^\circ\text{C} + 11.5\text{L}:12.5\text{D}$ (28.3%) and different temperature and photoperiod rampings (17.2%) were on par, while no mortality was observed in diapausing larvae under $27 \pm 1^\circ\text{C} + 12\text{L}:12\text{D}$ treatment conditions (table 6).

Supernumerary moults during diapause

During the course of diapause, each diapaused larvae exhibited several larval-larval ecdysis last being the larval-pupal ecdysis. We observed up to five supernumerary moults in the diapaused *C. partellus* larvae before final ecdysis to pupation. Highest diapausing larvae (35.7%) exhibited two supernumerary moults followed by 25.0% twice, 21.4% once, 14.2% four times, and 3.5% larvae moulted five times before final ecdysis to pupation (fig. 1).

Discussion

Intensity of diapause is genetic and physiological attributes are governed by the duration of diapause under given environmental conditions (Masaki, 2002; Jiang *et al.*, 2010). Tropical and sub-tropical insects undergo facultative diapause induced by environmental cues such as photoperiod, temperature, humidity etc., and require certain period for development (Wu, 2002; Sinclair *et al.*, 2003). Such environmental cues have also been reported to induce diapause in *B. fusca* (Usua, 1973; Dejen *et al.*, 2014), *Chilo* spp. (Scheltes, 1978), *Diatraea grandiosella* Dyar (Kikukawa & Chippendale, 1983), and *Scirpophaga incertulas* (Walker) (Gu & Yin, 1984). Present studies showed that the induction of diapause in *C. partellus* larvae takes from 46 to 56 days depending upon the temperature and photoperiod

Table 5. Duration of developmental stages and degree-day requirement for the survival of non-diapausing and diapausing *Chilo partellus* larvae at constant treatments.

Temperature (°C) and photoperiod (L:D) treatments	Larval duration (days)		Pupal duration (days)		Thermal requirement (Degree-days)	
	Non-diapause	Diapause	Non-diapause	Diapause	Non-diapause	Diapause
27 (12:12)	39.6 ± 0.87Aa	94.9 ± 2.32Ab	9.9 ± 0.16Aa	13.7 ± 0.80Ab	1067.9 ± 23.1Aa	2561.7 ± 13.28Ab
22 (11.5:12.5)	66.5 ± 0.66Ba	127.4 ± 3.42Bb	11.8 ± 0.18Ba	18.0 ± 0.88Bb	1462.2 ± 14.72Ba	2804.9 ± 31.15Bb
For comparing Treatments	LSD (<i>P</i> = 0.05)		LSD (<i>P</i> = 0.05)		LSD (<i>P</i> = 0.05)	
Larval state	2.304		0.96		53.29	
Treatment × Larval state	2.304		0.96		53.29	
	3.258		1.36		73.36	

Values within column and rows for each parameter followed by different capital and small letters, respectively are significant at *P* = 0.05.

Table 6. Initiation and termination of diapause in *Chilo partellus* at various temperature and photoperiod treatment regimes.

Treatments	Diapause induction (%)	Population loss due to diapause (%)
27 ± 1°C + 12L:12D	7.9 ± 0.92b	0.0 ± 0.00b
22 ± 1°C + 11.5L:12.5D	15.0 ± 1.50a	28.3 ± 6.00a
Temperature and photoperiod rampings	18.3 ± 0.83a	17.2 ± 4.70a
LSD (<i>P</i> = 0.05)	5.31	12.34

Values within columns for each parameter followed by different letters are significant at *P* = 0.05.

treatments. Induction of hibernation in *C. partellus* varied from 15.0 to 18.3% across temperature and photoperiod regimes, indicating that not all larvae undergo diapause, which could be due to genetic segregation. Induction of hibernation in some *C. partellus* larvae under ambient temperature and photoperiod conditions (27 ± 1°C + 12L:12D) during present studies could be due to synchrony between larval stage and physical and/or nutritional condition of the diet (as the larvae were reared on the same diet till pupation). These observations suggest substantial contribution of food along with other abiotic factors in induction of diapause in *C. partellus*. Further, the *C. partellus* larvae exposed to constant temperature and photoperiod treatments *viz.*, 18 ± 1°C + 11L:13D, 14 ± 1°C + 10.5L:13.5D and 10 ± 1°C + 10L:14D did not allow the larvae to reach maturity leading to 100% mortality without any symptoms of hibernation, indicating that the induction of diapause is triggered towards maturity of the larvae. Dry condition of the host plants and deterioration of nutritional quality, *i.e.*, increase in carbohydrate content and decrease in protein and water content in host plant have also been reported earlier to induce diapause in several cereal stem borer species even under favorable climatic conditions (Scheltes, 1978; Sinclair *et al.*, 2003; Tamiru *et al.*, 2012). *Chilo partellus* larvae exposed to constant 27 ± 1°C + 12L:12D presumed lower larval duration, higher larval survival, shorter pupal period, lower pupal mortality, and higher adult emergence as compared with those exposed to 22 ± 1°C + 11.5L:12.5D, reiterates that 27 ± 1°C temperature and 12L:12D photoperiod conditions are ambient for mass rearing of *C. partellus* under laboratory conditions.

During diapause *C. partellus* larvae exhibit several morphological changes, which aid in determining induction of diapause. We found that the morphological characteristics based criteria is more pronounced, reliable, and technically

sound as compared with behavioral and physiological criteria for diapause determination. The morphological changes in *C. partellus* larvae could be due to physiological changes in response to unfavorable abiotic factors and food quality. Earlier studies have also reported that the diet deficient in protein content affect growth and development, and influence cuticular pigmentation leading to transformation of spotted morph to unspotted diapausing morph in *D. grandiosella* (Chippendale & Reddy, 1973). Abiotic factors like cooling and freezing, and rates of temperature change influence physiological responses of insects (Sinclair *et al.*, 2003; Tamiru *et al.*, 2012), leading to effects on morphological traits such as larval weight, length and head capsule width. Present studies revealed that the larval weight, body length, and head capsule width of both diapausing and non-diapausing *C. partellus* larvae were significantly higher during temperature and photoperiod ramping 4 than other lower ramping regimes, which could be due to increased consumption and assimilation of food and prolonged larval duration. Further, the diapausing *C. partellus* larvae had significantly lower larval weight, larval length and head capsule width, and longer larval and pupal durations than the non-diapausing larvae at 27 ± 1°C + 12L:12D and 22 ± 1°C + 11.5L:12.5D treatment conditions. The reduction in body weight and length of diapausing *C. partellus* larvae could be because of reduced feeding, energy loss due to construction of diapause chamber, and reduction in body water content. Similar causal factors have also been reported earlier for reduction in weight and size of other diapausing insect species (Scheltes, 1978; Kostal *et al.*, 1998; Singtripop *et al.*, 1999). Furthermore, Koidsumi & Makino (1958) elaborated that the termination of hibernation in *C. suppressalis* is greatly accelerated by increase of water content in the body, resulting in the speeding up of certain enzymatic activities related to metamorphosis, and this increased water content is not expressed as increase in body weight but to serve for the production of metabolic water.

Insects adjust their life cycles to the seasonally variable environmental conditions in such a way that their growth, development, and reproduction coincide with favorable conditions (Wipking, 2000; Dhillon & Sharma, 2007; Khadioli *et al.*, 2014), and diapause is one such important component, which help insects to overcome and adjust variable climatic conditions. Induction and termination of diapause is regulated by temperature, which can simply be explained in terms of degree-days. The *C. partellus* larvae undergoing hibernation process under 27 ± 1°C + 12L:12D and 22 ± 1°C + 11.5L:12.5D constant temperature and photoperiod treatment conditions required 2561.7 and 2804.9 degree-days, respectively for completion of larval development, suggesting that the thermal

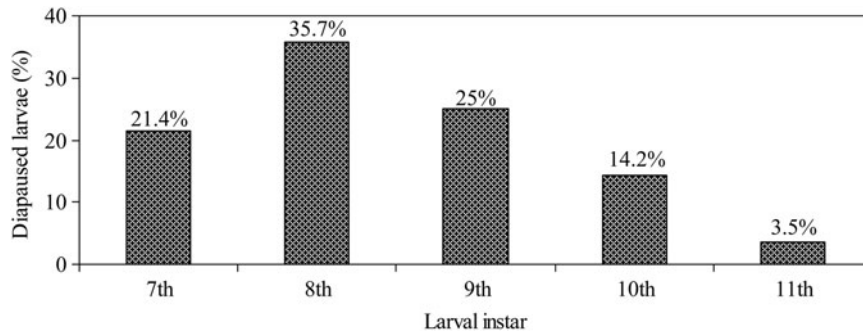


Fig. 1. Frequency distribution of number of supernumerary moults by diapausing *Chilo partellus* larvae.

requirement for diapausing *C. partellus* larvae (right from initiation to termination) decrease with increase in temperature. However, under fluctuating treatment (ramping) conditions the diapausing larvae required 3139.3 degree-days for completion of development, indicating influence of below optimum fluctuation in temperature and photoperiod conditions on the physiological response thus disrupting growth and development, and hence accumulating more numbers of degree-days (Wilson & Barnett, 1983; Khadioli *et al.*, 2014).

The number of larval ecdysis in normal growing larvae of *C. partellus* is genetically fixed to six (Nijhout, 1975). However, inter- and intra-specific variation in number of supernumerary moults in the diapausing insects cannot be ignored. This inter- and intra-specific variation in supernumerary moults could be because of food (Pipa, 1976), environmental conditions (Kadono-Okuda *et al.*, 1986), or juvenile hormone activity (Yin & Chippendale, 1973; Tanaka & Takeda, 1993). During present studies we observed up to five supernumerary moults in the hibernating *C. partellus* larvae, wherein highest percentage of larvae (35.7%) exhibited two supernumerary moults followed by 25.0% twice, 21.4% once, 14.2% four times, and 3.5% larvae moulted five times before final ecdysis to pupation. This variation in supernumerary ecdysis in *C. partellus* could be due to variation in active forms of juvenile hormone titers during the entire course of diapause. Furthermore, the diapausing *C. partellus* larvae undergoing supernumerary or stationary moults exhibited reduction in larval weight and size, indicating that the supernumerary moults does not seem to provide any adaptive advantage to insects. Earlier studies reported seven supernumerary moults in diapausing *C. partellus* larvae (Kfir, 1991) and two additional moults in *D. grandiosella* larvae (Chippendale & Yin, 1973; Yin & Chippendale, 1976) with stationary larval growth.

Feeding activities in the beginning and end of hibernation period are considered to serve for the nutritive preparations for entering into and termination of hibernation in *C. suppressalis*, and the environmental conditions determine the feeding activity, diapause duration, and successful diapause termination (Koidsumi & Makino, 1958; Fischer *et al.*, 2014). During present studies, the termination of diapause in *C. partellus* larvae occurred between 8 and 12 days on exposure to ambient conditions, and completed their development right from induction of diapause to pupation between 94.9 and 160.4 days. The *C. partellus* larvae undergoing diapause under different temperature and photoperiod conditions took 52.4–60.9 more number of days than the non-diapausing larvae to complete the development, suggesting that the correct

combination of temperature and photoperiod is highly desirable for termination and post-diapause development. The pupal period exceeded from 3.2 to 6.2 days in the diapausing *C. partellus* larvae under different temperature and photoperiod conditions as compared with non-diapausing larvae, indicating significant effect of hibernation on development of adult characteristics during pupal stage and pupa-adult metamorphosis. Survival of *C. partellus* larvae undergoing diapause is critical for population pressure buildup on resumption of ambient conditions and damage threshold in the target host crop. Temperature range from 25 to 32°C is suitable for overwintering larvae of *C. partellus* to terminate diapause (Jalali & Singh, 2006). A few *C. partellus* larvae underwent diapause under ambient conditions ($27 \pm 1^\circ\text{C} + 11.5\text{L}:12.5\text{D}$) could be due to genetic segregation for diapause and some other unknown reasons, but no population loss was observed due to diapause under these conditions. Further, a population loss of 17.2–28.3% was recorded during hibernation process across different temperature and photoperiod conditions, which is significant number to determine population buildup of first brood after termination of hibernation and has implications for devising appropriate strategies for the management of *C. partellus*.

Acknowledgements

The technical and laboratory assistance of Mr. Brajendarnath and Ms. Prachi Tyagi, and funding by Ministry of Science and Technology, Department of Science and Technology (DST), New Delhi, India (SERB No:SB/SO/AS-020/2013) is gratefully acknowledged. The authors also sincerely thank the editor and five anonymous reviewers for constructive comments to improve quality of the manuscript.

References

- Andrewartha, H.G. (1952) Diapause in relation to the ecology of insects. *Biological Reviews* 27, 50–107.
- Arbab, A. (2014) Spatial distribution and minimum sample size for overwintering larvae of the rice stem borer *Chilo suppressalis* (Walker) in paddy fields. *Neotropical Entomologists* 43, 415–420.
- Beck, S.D. (1980) *Insect Photoperiodism*. 2nd ed. New York, Academic Press.
- Chippendale, G.M. & Reddy, A.S. (1973) Temperature and photoperiod regulation of diapause of the southwestern corn

- borer *Diatraea grandiosella*. *Journal of Insect Physiology* **19**, 1397–1408.
- Chippendale, G.M. & Yin, C.M.** (1973) Endocrine activity retained in diapause insect larvae. *Nature* **246**, 511–513.
- Dejen, A., Getu, E., Azerefeagne, F. & Ayalew, A.** (2014) Distribution and impact of *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) in Northeastern Ethiopia. *Journal of Entomology and Nematology* **6**, 1–13.
- Dhaliwal, G.S., Jindal, V. & Mohindru, B.** (2015) Crop losses due to insect pests: global and Indian scenario. *Indian Journal of Entomology* **77**(2), 165–168.
- Dhillon, M.K. & Sharma, H.C.** (2007) Effect of storage temperature and duration on viability of eggs of *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Bulletin of Entomological Research* **97**, 55–59.
- Ellers, J. & van Alphen, J.J.M.** (2002) A trade-off between diapause duration and fitness in female parasitoids. *Ecological Entomology* **27**, 279–284.
- Fischer, K., Klockmann, M. & Reim, E.** (2014) Strong negative effects of simulated heat waves in a tropical butterfly. *Journal of Experimental Biology* **217**, 2892–2898.
- Gadenne, C., Dufour, M.C., Rossignol, F., Bizard, J.M. & Franck, C.** (1997) Occurrence of non-stationary larval moults during diapause in the corn stalk borer, *Sesamia nonagrioides* (Lepidoptera: Noctuidae). *Journal of Insect Physiology* **43**, 425–431.
- Gu, D.J. & Yin, J.K.** (1984) Preliminary study on the termination of larval diapause in paddy borer, *Scirpophaga incertulas* (Walker). *Journal of South China Agricultural University* **4**, 65–73.
- Hodek, I.** (2012) Adult diapause in Coleoptera. *Psyche* **2012**, 1–10.
- Irwin, J.T. & Lee, R.E.** (2000) Mild winter temperature reduces survival and potential fecundity of the goldenrod gall fly, *Eurosta solidaginis* (Diptera: Tephritidae). *Journal of Insect Physiology* **46**, 655–661.
- Jalali, S.K. & Singh, S.P.** (2006) Biological control of *Chilo partellus* using egg parasitoid *Trichogramma chilonis* and *Bacillus thuringiensis*. *Indian Journal of Agricultural Research* **40**, 184–189.
- Jiang, X.F., Cao, W.J., Zhang, L. & Luo, L.Z.** (2010) Beet webworm (*Loxostege sticticalis* Linnaeus) (Lepidoptera: Pyralidae) migration in China: evidence from genetic markers. *Environmental Entomology* **39**, 232–242.
- Kadono-Okuda, K., Kajitara, Z. & Yamashita, O.** (1986) Induction of perfect superlarvae by the application of juvenile hormone analogue to starved larvae of the silkworm, *Bombyx mori*. *Journal of Insect Physiology* **32**, 1065–1073.
- Kfir, R.** (1991) Effect of diapause on development and reproduction of the stem borers *Busseola fusca* (Lepidoptera: Noctuidae) and *Chilo partellus* (Lepidoptera: Pyralidae). *Journal of Economic Entomology* **84**, 1677–1680.
- Kfir, R.** (1993) Diapause termination in *Busseola fusca* (Lepidoptera: Noctuidae) in the laboratory. *Annals of Entomological Society of America* **86**, 273–277.
- Kfir, R., Overholt, W.A., Khan, Z.R. & Polaszek, A.** (2002) Biology and management of economically important lepidopteran cereal stem borers in Africa. *Annual Review of Entomology* **47**, 701–731.
- Khadioli, N., Tonnang, Z.E.H., Muchugu, E., Ong'amo, G., Achia, T., Kipchirchir, I., Krosche, J. & Le Ru, B.** (2014) Effect of temperature on the phenology of *Chilo partellus* (Swinhoe) (Lepidoptera, Crambidae); simulation and visualization of the potential future distribution of *C. partellus* in Africa under warmer temperatures through the development of life-table parameters. *Bulletin of Entomological Research* **104**, 809–822.
- Kikukawa, S. & Chippendale, G.** (1983) Seasonal adaptations of populations of the southwestern corn borer, *Diatraea grandiosella*, from tropical and temperate regions. *Journal of Insect Physiology* **29**, 561–567.
- Koidsumi, K. & Makino, K.** (1958) Intake of food during hibernation of the rice stem borer, *Chilo suppressalis* Walker. *Japanese Journal of Applied Entomology and Zoology* **2**, 135–138.
- Kostal, V., Sula, J. & Simek, P.** (1998) Physiology of drought tolerance and cold hardiness of the Mediterranean tiger moth *Cymbalophora pudica* during summer diapause. *Journal of Insect Physiology* **44**, 165–173.
- Lehmann, P., Lyytinen, A., Piironen, S. & Lindström, L.** (2015) Latitudinal differences in diapause related photoperiodic responses of European Colorado potato beetles (*Leptinotarsa decemlineata*). *Evolutionary Ecology* **29**, 269–282.
- Masaki, S.** (2002) Ecophysiological consequences of variability in diapause intensity. *European Journal of Entomology* **99**, 143–154.
- Mathez, F.C.** (1972) *Chilo partellus* Swinh., *C. orichalcociliellus* Strand (Lep., Crambidae) and *Sesamia calamistis* Hmps. (Lep., Noctuidae) on maize in the Coast Province, Kenya. *Bulletin de la Société Entomologique Suisse* **45**, 267–289.
- Neal, J.W., Chittams, J.L. & Bentz, J.** (1997) Spring emergence by larvae of the eastern tent caterpillar (Lepidoptera: Lasiocampidae): a hedge against high-risk conditions. *Annals of Entomological Society of America* **90**, 596–603.
- Nijhout, H.F.** (1975) A threshold size for metamorphosis in the tobacco horn worm, *Manduca sexta*. *Biological Bulletin* **149**, 214–225.
- Ofomata, V.C., Overholt, W.A. & Egwuatu, R.I.** (1999) Diapause termination of *Chilo partellus* (Swinhoe) and *Chilo orichalcociliellus* Strand (Lepidoptera: Pyralidae). *Insect Science and its Applications* **19**, 187–191.
- Pipa, R.L.** (1976) Supernumerary instars produced by chilled wax moth larvae: endocrine mechanisms. *Journal of Insect Physiology* **22**, 1641–1647.
- Qiang, C.K., Du, Y.Z., Qin, Y.H., Yu, L.Y., Zhou, B.Y., Feng, W.J. & Wang, S.S.** (2012) Overwintering physiology of the rice stem borer larvae, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae): roles of glycerol, amino acids, low-molecular weight carbohydrates and antioxidant enzymes. *African Journal of Biotechnology* **66**, 13030–13039.
- Roe, R.M., Hammond, A.M., Douglas, E.E. & Philogene, B.J.R.** (1984) Photoperiodically induced delayed metamorphosis in the sugarcane borer, *Diatraea saccharalis* (Lepidoptera: Pyralidae). *Annals of Entomological Society of America* **71**, 312–318.
- Scheltes, P.** (1978) The condition of the host plant during aestivation diapause of the stalk borers *Chilo partellus* and *Chilo orichalcociliella* (Lepidoptera: Pyralidae) in Kenya. *Entomologia Experimentalis et Applicata* **24**, 479–488.
- Sharma, H.C., Taneja, S.L., Leuschner, K. & Nwanze, K.F.** (1992) Techniques to screen sorghum for resistance to insect pests. *Information Bulletin* No. 32, 48 pp. International Crops Research Institute for the Semi-Arid Tropics, Patancheru 502 324, Andhra Pradesh, India.
- Sinclair, B.J., Vernon, P., Kloke, C.J. & Chown, S.L.** (2003) Insects at low temperatures: an ecological perspective. *Trends in Ecology and Evolution* **18**, 257–262.
- Singtripop, T., Wanichacheewa, S., Tsuzuki, S. & Sakurai, S.** (1999) Larval growth and diapause in a tropical moth *Omphisa fuscidentalis* Hampson. *Zoological Science* **16**, 725–733.
- Sotherlind, L. & Nylin, S.** (2011) Genetics of diapause in the comma butterfly, *Polygonia c-album*. *Physiological Entomology* **36**, 8–13.

- Tamiru, A., Getu, E., Jembere, B. & Bruce, T.** (2012) Effect of temperature and relative humidity on the development and fecundity of *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae). *Bulletin of Entomological Research* **102**, 9–15.
- Tanaka, Y. & Takeda, S.** (1993) Ultrannumerary larval ecdyses of the silkworm, *Bombyx mori* induced by ecdysone. *Nature* **80**, 131–132.
- Tauber, M.J., Tauber, C.A. & Masaki, S.** (1986) *Seasonal Adaptations of Insects*. New York, Oxford University Press.
- Usua, E.J.** (1973) Induction of diapause in the maize stem borer, *Busseola fusca*. *Entomologia Experimentalis et Applicata* **16**, 322–328.
- Wilson, L.T. & Barnett, W.W.** (1983) Degree-days: an aid in crop and pest management. *California Agriculture (USA)* **37**, 4–7.
- Wipking, W.** (2000) Survival in space and time: flexibility and variability guarantee reproductive success in the burnet moth *Zygaena trifolii*. *Mitteilungen-der-Deutschen-Gesellschaft-fur-allgemeine-und-angewandte-Entomologie* **12**, 595–598.
- Wu, K.J.** (2002) A consideration about dormancy in relation to diapause in insects. *Entomological Knowledge* **39**, 154–160.
- Xiao, H.J., Mou, F.C., Zhu, X.F. & Xue, F.S.** (2010) Diapause induction, maintenance and termination in the rice stem borer, *Chilo suppressalis* (Walker). *Journal of Insect Physiology* **56**, 1558–1564.
- Yin, C.M. & Chippendale, G.M.** (1973) Juvenile hormone regulation of the larval diapause of the southwestern corn borer, *Diatraea grandiosella*. *Journal of Insect Physiology* **19**, 2403–2420.
- Yin, C.M. & Chippendale, G.M.** (1976) Hormonal control of larval diapause and metamorphism of the southwestern corn borer *Diatraea grandiosella*. *Journal of Experimental Biology* **64**, 303–310.
- Youcum, G.D., Rinehart, J.P. & Larson, M.L.** (2011) Monitoring diapause development in the Colorado potato beetle, *Leptinotarsa decemlineata*, under field conditions using molecular biomarkers. *Journal of Insect Physiology* **57**, 645–652.