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Hibernation induced biochemical changes in spotted stem borer *Chilo partellus*

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ABSTRACT

Spotted stem borer, *Chilo partellus* (Swinhoe) is one of the most destructive pests of sorghum and maize, and undergoes hibernation during harsh winters. Several aspects of diapause have been studied, however metabolic and biochemical processes underlying hibernation remain poorly understood in *C. partellus*. Present studies carried out during 2020–22 at ICAR-Indian Agricultural Research Institute, New Delhi deciphered the variations in nutritional biochemicals, digestive and stress enzymes, cryoprotectants in the hibernation and in non-hibernating larvae and pupae of *C. partellus*. These studies revealed that the total lipids and proteins were significantly greater during pre-hibernation and hibernation, and sugars were greater in non-hibernation larvae. Glycogen content was greater in pre-hibernation, and sorbitol, trehalose and glucitol contents consistently increased from pre-hibernation to hibernation stages in comparison to non-hibernation larvae of *C. partellus*. Total sugar and sorbitol contents decreased, while total protein, trehalose and glucitol increased, in post-hibernation than the non-hibernation pupae of *C. partellus*. Activities of ascorbic acid, lipid peroxidation, total antioxidant, catalase and superoxide dismutase were significantly greater during hibernation followed by pre-hibernation as compared to non-hibernation larvae, while the reverse was the case with protease activity. Ascorbic acid, lipid peroxidation, total antioxidant and catalase activities were greater in post-hibernation as compared to non-hibernation pupae of *C. partellus*. However, glutathione S-transferase activity was greater in the non-hibernation larvae and pupae than the hibernation larvae and post-hibernation pupae of *C. partellus*. These findings can be useful to design newer management strategies keeping in view the weak links like the state of nutritional metabolism and oxidative stress tolerance due to diapause in *C. partellus*.

Keywords: *Chilo partellus*, Cryoprotectants, Hibernation, Nutritional compounds, Stress enzymes

Insects encounter numerous environmental stresses, and their survival and development are regulated by these factors. However, in response to the stresses, insects have developed various mechanisms like diapause to survive harsh environmental conditions. Diapause in many insect species has evolved as a mechanism to survive under predictable adverse climatic conditions. The diapause process is an endocrine controlled, genetically regulated physiological state of arrested metabolic activity (Dhillon *et al.* 2020). Diapause, according to the season of its occurrence, is generally classified as winter diapause: hibernation and summer diapause: aestivation. During diapause the insect passes through several phases like diapause induction, maintenance, termination, and post-diapause development undergoing specific biochemical and physiological changes. The biochemical changes that occur during diapause have important ecological and physiological implications, particularly in terms of how metabolic reserves are allocated and used to produce other necessary metabolites during distinct phases of hibernation (Sadakiyo and Ishihara 2012).

The spotted stem borer, *Chilo partellus* (Swinhoe) is considered to be one of the most destructive pests causing 18–25% yield losses in sorghum and maize in tropical and temperate areas in Asia and Africa. The important feature of *C. partellus* biology is the arrangement of the facultative type of larval diapause. The north Indian population of *C. partellus* enters into hibernation (Dhillon *et al.* 2017). Several aspects of diapause have been investigated environmental factors determining diapause (Dhillon *et al.* 2017, Dhillon *et al.* 2019 a,b), temperature dependent development (Dhillon and Hasan 2017), consequences of diapause on reproductive physiology (Dhillon and Hasan 2018, Dhillon *et al.* 2019b), genetic regulation of diapause in *C. partellus* (Dhillon *et al.* 2020). However, the stage specific biochemical characteristics of overwintering *C. partellus* remain poorly understood. Specifically, little is known about whether hibernation results in changes to key biomolecules such as sugars, lipids, protein, enzymes, glycogen, sorbitol, trehalose, and glucitol. To address this gap, we investigated the dynamic profiles of several biochemical parameters like total lipids, protein, sugars, different digestive and stress enzymes, glycogen, trehalose,

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sorbitol, and glucitol in the hibernation and non-hibernation larvae and pupae of *C. partellus*. Our study aimed to shed light on the metabolic and biochemical processes underlying hibernation in *C. partellus*, and to provide insights into the overwintering strategy of this pest. These findings will deepen our understanding of the metabolic strategy of *C. partellus* to counter stressful environmental conditions during overwintering.

MATERIALS AND METHODS

Maintenance of stock culture and induction of hibernation in C. partellus: The *C. partellus* larvae were collected from maize crops raised at experimental fields of Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana during 2020, and further studies were carried out during 2020–22. These larvae were initially reared on maize stalks till pupation under ambient insect rearing conditions (27 ± 1°C, 70 ± 5% RH and 12 L:12D) in walk-in Insect growth chambers (RINAC) at Division of Entomology, ICAR-Indian Agricultural Research Institute, New Delhi. Adults obtained from these pupae were paired in oviposition cages, and the eggs obtained were collected daily and kept in separate plastic jars provided with moist cotton at the bottom. Freshly hatched *C. partellus* larvae were thereafter maintained on an artificial diet (Sharma *et al.* 1992), and the culture was maintained around the year under ambient insect-rearing laboratory conditions (27 ± 1°C, 70 ± 5% RH and 12 L:12D).

The late fourth to early fifth instar *C. partellus* larvae (16–18 days old) obtained from the aforesaid laboratory culture were exposed to hibernation induction treatments (exposure at weekly intervals at 27±1ºC + 12L:12D, 22±1ºC + 11.5L:12.5D, 18±1ºC + 11L:13D, 14±1ºC + 10.5L:13.5D, and 10±1ºC + 10L:14D) as per the method given by Dhillon *et al.* (2017). One month after hibernation induction treatment, the *C. partellus* larvae showing pre-hibernation symptoms like smaller in body size, disappearing of cuticular lining and dark spots, and start of constructing diapause chamber in artificial diet were collected as pre-hibernation stage. The *C. partellus* larvae showing diapause symptoms such as complete loss of markings, spots, asetose tubercle, cuticular pigmentation, and turning of larval body from creamy to milky white were considered to be in hibernation, and at 40 days after this stage (around 3-month-old larvae), the larvae were collected in the glass vials. Thereafter, the remaining hibernating larvae were exposed to hibernation termination treatment conditions, i.e. fresh diet and at 27 ± 1°C, 70 ± 5% RH and 12 L:12 D, to obtain the pupae from hibernation treatment. One set of larvae was kept under ambient rearing conditions, and the counterpart insect stages (larvae and pupae) were collected as non-hibernation controls. Finally, the test samples of each larval (non-hibernation, pre-hibernation and hibernation), and pupal (non-hibernation and post-hibernation) stages of *C. partellus* were collected in three sets (each having 15–20 insects) in glass vials and stored at -80°C in the deep freezer (Panasonic, India), and used in the present studies. There were three replications for each biochemical parameter in a completely randomized design.

Estimation of total lipids, proteins and sugars: The total lipids in the test *C. partellus* samples were estimated using Sulpho-Phospho-Vanillin method (Barnes and Blackstock 1973), and the values were expressed as mg/g of fresh weight. Total protein content in the samples was determined using the method of Bradford (1976), having Bovine Serum Albumin as a standard, and values expressed as mg/g of fresh weight. Total sugars in test *C. partellus* samples were estimated by phenol-sulphuric acid method (Dubois *et al.* 1956), using glucose as standard, and expressed as mg/g of fresh weight.

Estimation of glycogen and cryoprotectants: Glycogen extraction was done as per Goto *et al.* (1993), estimated by the phenol-sulfuric acid method (Dubois *et al.* 1956), and expressed as mg/g of insect tissue. The levels of cryoprotectant biomolecules namely trehalose, sorbitol, and glucitol in test *C. partellus* samples were measured using the method by Kostal and Simek (1996). The o-methyloxime trimethysilyl derivatives of cryoprotectants present in samples were analyzed using capillary gas chromatography. Aliquot of 1 µl was injected into a split injection port (split ratio 20:1) of a gas chromatograph (GCMS-QP2010, Shimadzu). Separation and quantitation of sorbitol, trehalose, and glucitol were achieved on Rtx®-5MS column. Injection, interface, and ion source temperatures were set at 300, 300 and 270ºC, respectively. The temperature program was set at 180°C then 10°C/min to 300°C and 3 min hold. Chromatograms and mass spectra were evaluated using the Labsolutions® GCMS software version 2.71 (Shimadzu). The cryoprotectant compounds were identified using MS libraries (NIST08, Wiley8) from different chromatogram peaks and expressed as µg/mg.

Estimation of different enzymes: Ascorbic acid in the test *C. partellus* samples was estimated using the method given by Zannoni *et al.* (1974), and values were expressed as mg/g of insect sample. Protease activity was measured using azocasein method by Gatehouse *et al.* (1999), absorbance measured at 440 nm, values calculated based on the extinction coefficient of azocasein (35/mm/cm), and expressed as µmol/ml/min of the sample extract. Lipid peroxidation was measured by the method given by Cakmak and Horst (1991), with absorbance measured at 532 and 600 nm, values calculated based on the extinction coefficient of 156/mm/cm, and were expressed as µmol/ml/min of the sample extract.

Total antioxidant content was determined using the method developed by Prieto *et al.* (1999), and values expressed as mg/g of insect tissue. Glutathione S-transferase activity was measured using 1-chloro-2,4-dinitrobenzene as a substrate as per the method given by Habig *et al.* (1974) at 340 nm wavelength, and values expressed as µmol/ml/min of the sample extract. The catalase activity was measured by the method given by Sinha (1972), and values were expressed as µmol/ml/min of the sample extract. The superoxide dismutase activity was determined according

to the method of Dhindsa *et al.* (1981), by measuring the reduction in absorbance caused by the enzyme's antioxidant activity and expressed in units/ml of sample.

Statistical analysis: To compare the differences in biochemical profiles of pre-hibernation, hibernation and nondiapause *C. partellus* larvae, the tests were conducted in three replications in a completely randomized design. Raw data of different biochemical compounds from various stages of *C. partellus* strains were subjected to one-way analysis of variance. The significance of differences was checked by F-test, and treatment means were compared using least significant differences at P = 0.05 by using the statistical software R®.

RESULTS AND DISCUSSION

Variation in nutritional biochemical constituents during different stages of hibernation and non-hibernation in C. partellus: The amounts of total lipids ($F_{2,6}$=395.37, P<0.001), proteins ($F_{2,6}$=53.82, P<0.001), and sugars ($F_{2,6}$=119.99, P<0.001) significantly varied among the *C. partellus* larvae from pre-hibernation, hibernation and non-hibernation stages (Fig 1). Total lipids and proteins were significantly greater during pre-hibernation followed by hibernation, and were greater in these stages as compared to the non-hibernation larvae, indicating their probable roles in the maintenance of hibernation in *C. partellus*. Earlier studies also recorded increased lipid accumulation during pre-hibernation till the onset of diapause, which then started declining towards the end of diapause in pistachio seed wasp, *Eurytoma plotnikovi* Nikol'skaya (Mohammadzadeh *et al*. 2017). Total protein was also found to be increased during hibernation in the haemolymph in the case of ladybird, *Harmonia axyridis* (Pallas) (Rericha *et al*. 2021). However, the total sugars were significantly greater in non-hibernation in comparison to pre-hibernation and hibernation stages, suggesting that the total sugars start declining in the process of hibernation in *C. partellus* (Fig 1). Conversely, total sugar content was reported to be increased during hibernation compared to non-hibernation larvae in the case of pistachio seed wasp, *E. plotnikovi* (Mohammadzadeh *et al.* 2017). The total proteins ($F_{1,4}$=36.73, P=0.004) and total sugars ($F_{1,4}$=302.32, P<0.001) also varied significantly among the post-hibernation and non-hibernation pupae of *C. partellus*, while the differences for total lipids were nonsignificant (Fig 1). The greater the total proteins and lower sugars in the post-hibernation than the non-hibernation pupae suggest that the additional accumulated proteins in hibernation larvae might have passed on to pupae for successful metamorphosis into adults, and the least requirement of sugars in this process (Tanwar *et al.* 2021).

Insects frequently produce and store cryoprotectants during their overwintering period. The accumulated low molecular-weight carbohydrates such as sorbitol, trehalose, and glucose regulate cold tolerance during hibernation (Saeidi and Moharramipour 2017, Hasanvand *et al.* 2020). The contents of glycogen ($F_{2,6}$=1228.20, P<0.001), sorbitol ($F_{2,6}$=546.51, P<0.001), trehalose ($F_{2,6}$=570.04, P<0.001), and glucitol ($F_{2,6}$=1620.20, P<0.001) significantly differed among the pre-hibernation, hibernation and non-hibernation stages of the *C. partellus* larvae (Table 1). The greater glycogen content during the pre-hibernation in comparison to hibernation and non-hibernation stages suggest that the glycogen act as shuttling source of energy to fuel the initiation of hibernation process in the larvae of *C. partellus* as suggested in other insects by Goto *et al.* (1993). However, there was consistent and significant increase in sorbitol, trehalose and glucitol contents from pre-hibernation to hibernation in comparison to non-hibernation larvae

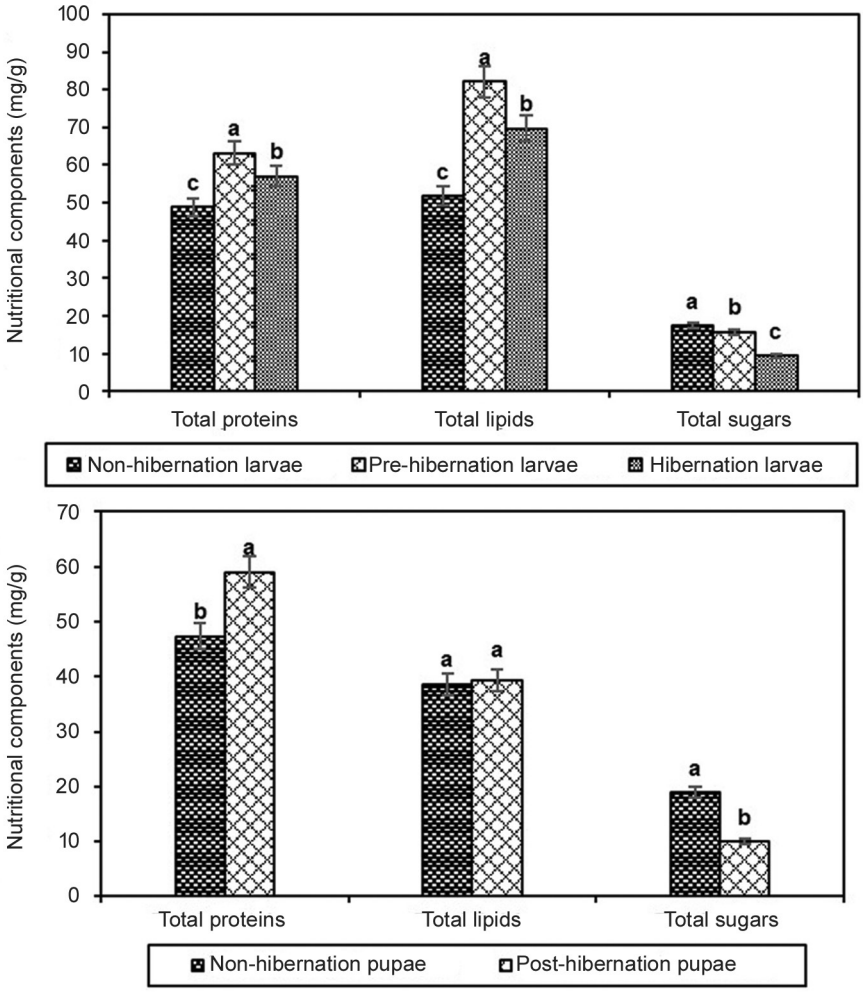

Fig 1 Amount of total proteins, total lipids and total sugars in different stages of larvae and pupae of hibernation and non-hibernation *Chilo partellus.*

Table 1 Amount of glycogen, sorbitol, trehalose and glucitol in different stages of larvae and pupae of hibernation and non-hibernation *Chilo partellus*

Life stages	Glycogen (mg/g)	Sorbitol (µg/mg)	Trehalose (µg/mg)	Glucitol (µg/mg)
Larvae				
Non-hibernation	6.14	0.61	1.45	9.94
Pre-hibernation	8.46	2	1.42	15.2
Hibernation	4.63	8.98	2.57	22.02
F-probability	<0.001	<0.001	<0.001	<0.001
LSD (P = 0.05)	0.19	0.66	0.1	0.52
Pupae				
Non-hibernation	4.51	8.23	1.4	12.46
Post-hibernation	5.22	6.84	1.77	16.85
F-probability	0.093	0.019	0.006	<0.001
LSD (P = 0.05)	NS	1.02	0.19	0.36

NS, Values non-significant at P = 0.05.

(Table 1). Earlier studies also reported decline in glycogen with the initiation of hibernation, which was proportional to increase in sorbitol and trehalose contents in *E. plotnikovi* (Mohammadzadeh *et al.* 2017). Thus, present findings suggest that the biomolecules like sorbitol, trehalose and glucitol support the process as well as maintenance of hibernation, and act as cryoprotectants for the *C. partellus* larvae to survive under chilling winter conditions as in case of *Eurygaster integriceps* Puton by Hasanvand *et al.* (2020). There were significant differences in the sorbitol ($F_{1,4}$=14.45, P=0.019), trehalose ($F_{1,4}$=28.47, P=0.006) and glucitol ($F_{1,4}$=1125.80, P<0.001) contents in the non-hibernation and post-hibernation pupae of *C. partellus*, while the differences for glycogen content were nonsignificant (Table 1). The sorbitol content decreased, while trehalose and glucitol increased in the post-hibernation than the non-hibernation pupae of *C. partellus* (Table 1). These findings indicate little role of sorbitol during pupal unlike larval hibernation stage in the *C. partellus*. However, the greater contents of trehalose and glucitol in post-hibernation than the non-hibernation pupae suggest their role to serve as additional source of energy to support and protect the post-hibernation pupae and successful metamorphosis of *C. partellus* into adults.

Expression of digestive and stress enzymes during different stages of hibernation and non-hibernation in C. partellus: The expression of digestive enzymes such as ascorbic acid ($F_{2,6}$=30.90, P<0.001), protease ($F_{2,6}$=895.72, P<0.001) and lipid peroxidation ($F_{2,6}$=20.07, P=0.002) significantly varied among the larvae from pre-hibernation, hibernation and non-hibernation stages in *C. partellus* (Table 2). The activities of ascorbic acid and lipid peroxidation were significantly greater during hibernation followed by pre-hibernation as compared to non-hibernation larvae of the *C. partellus* (Table 2). However, the ascorbic acid content was found decreased during hibernation in red mason bees, *Osmia bicornis* L. (Dmochowska-slezak *et al.* 2015). The reverse was the case with protease activity in the hibernation, pre-hibernation and non-hibernation larvae of *C. partellus* (Table 2). Present findings suggest that due to greater utilization of accumulated proteins, the protease content start declining in the larvae during the process of hibernation as compared to non-hibernation *C. partellus* (Table 2). The photoperiod has also been reported to influence the protease activity during the process of hibernation in fall webworm, *Hyphantria cunea* (Drury) (Zhao *et al.* 2021). However, the increased activities of ascorbic acid and lipid peroxidation during the hibernation as compared to pre-hibernation and non-hibernation stages suggest that the accumulated lipids continue to serve as source of energy during the process of hibernation in

Table 2 Activity of various enzymes in different stages of larvae and pupae of hibernation and non-hibernation *Chilo partellus*

Life stages	Ascorbic acid (mg/g)	Protease (µmol/ml/min)	Lipid peroxidation (µmol/ml/min)	Total antioxidant (mg/g)	Glutathione S-transferase (µmol/ml/min)	Catalase (µmol/ml/min)	Superoxide dismutase (U/ml)
Larvae							
Non-hibernation	0.11	3.04	2.04	1.26	355.63	4.45	0.80
Pre-hibernation	0.12	1.38	2.65	2.06	173.74	8.22	1.02
Hibernation	0.13	0.95	3.50	3.51	186.25	8.96	1.11
F-probability	<0.001	<0.001	0.002	<0.001	<0.001	<0.001	0.001
LSD (P = 0.05)	0.005	0.13	0.57	0.41	14.39	0.50	0.11
Pupae							
Non-hibernation	0.11	1.27	1.52	1.78	426.18	4.64	0.64
Post-hibernation	0.15	0.77	2.08	2.94	331.39	9.42	0.70
F-probability	<0.001	<0.001	0.002	0.013	<0.001	<0.001	0.695
LSD (P = 0.05)	0.004	0.07	0.22	0.74	8.98	0.83	NS

NS, Values non-significant at P = 0.05.

C. partellus (Table 2). Furthermore, the expression of ascorbic acid ($F_{1,4}$=727.77, P<0.001), protease ($F_{1,4}$=397.56, P<0.001) and lipid peroxidation ($F_{1,4}$=47.53, P=0.002) also varied significantly among the post-hibernation and non-hibernation pupae of *C. partellus* (Table 1). The increase in activities of ascorbic acid and lipid peroxidation in post-hibernation as compared to the non-hibernation pupae also suggest that the carryover lipids from the hibernation larvae continue to serve as energy source for successful metamorphosis into adults of *C. partellus* (Table 2). Greater levels of lipid peroxidation were also reported in the hibernation-destined pupae of the tropical tasar silkworm, *Antheraea mylitta* Drury (Sahoo *et al.* 2018).

Excessive accumulation of reactive oxygen species (ROS) can damage biological macromolecules, wherein antioxidant enzymes play a crucial role to mitigate the harmful effects of ROS. There was significant variation in stress enzymes like total antioxidant ($F_{2,6}$=91.73, P<0.001), glutathione S-transferase ($F_{2,6}$=596.70, P<0.001), catalase ($F_{2,6}$=281.74, P<0.001), and superoxide dismutase ($F_{2,6}$=24.26, P=0.001) among the larvae from pre-hibernation, hibernation and non-hibernation stages in *C. partellus* (Table 2). The total antioxidant, catalase and superoxide dismutase activities were significantly greater during hibernation followed by pre-hibernation as compared to non-hibernation larvae of the *C. partellus* (Table 2). This could be due to upregulation of these stress enzymes for synthesis of chilling tolerance biomolecules (Yang *et al.* 2013, Dmochowska-Slezak *et al.* 2015). The activities of catalase and superoxide dismutase were also found greater in diapause as compared to non-diapause larvae of *C. suppressalis* (Yang *et al.* 2013). The activity of stress enzymes like total antioxidant ($F_{1,4}$=18.56, P=0.013) and catalase ($F_{1,4}$=256.45, P<0.001) were also significantly greater in post-hibernation as compared to non-hibernation pupae of *C. partellus* (Table 2), indicating their continued support to the post-hibernation pupae to transform into adults of *C. partellus*. The glutathione S-transferase also varied significantly among the non-hibernation and post-hibernation pupae of *C. partellus* ($F_{1,4}$=859.66, P<0.001). However, the glutathione S-transferase activity was significantly greater in the non-hibernation larvae and pupae as compared to hibernation larvae and post-hibernation pupae of *C. partellus* (Table 2), indicating that the hibernating larvae and their pupae might result in reduced metabolism and greater toxicity due to diminished oxidative stress tolerance in cases where *C. partellus* gets exposed to the insecticides, as has also been observed in other studies (Mukesh K Dhillon, unpublished data).

Overall, the *C. partellus* larvae pass through several physiological phases and biochemical changes during the process of hibernation, and the information generated during the present studies on changes in nutritional components, cryoprotectants and associated stress enzymes will help in better understanding the nutritional biochemistry, weak links like the state of nutritional metabolism and oxidative stress tolerance during hibernation developmental biology, and design bait-based insecticides and/or identifying newer target sites for pesticide molecules for the management of *C. partellus*.

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