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Research Article
Starvation Modulates the Immuno-oxidative Stress and Cocoon Characteristics of Tasar Silkworm *Antheraea mylitta*

[1]J.P. Pandey, [1]K. Jena, [2]R.K. Tiwari and [1]Alok Sahay

[1]Central Tasar Research and Training Institute, Central Silk Board, Ministry of Textiles, Govt. of India, P.O. Piska-Nagri, 835303 Ranchi, India
[2]Department of Zoology, Kashi Naresh Government Post Graduate College, 221304 Gyanpur, S.R.N. Bhadohi, India

Abstract

Background and Objectives: Tasar silkworm is sericigenous insect which produces the unique quality silk. Loss occurring due to starvation is not quantified well at the level of immuno-oxidative and quantitative traits. Hence, the present study was designed to find out the effect of starvation on immuno-responses, oxidative damages, antioxidant defences and quantitative traits of tasar silkworm *Antheraea mylitta*. **Materials and Methods:** The 6 days old V instar larvae were subjected to starvation stress and impact was evaluated on morphology, protein concentration, enzyme activity, immunological parameters, oxidative stress and cocoon characteristics. **Results:** A change in total hemocyte count and differential hemocyte count indicates its maintenance of homeostasis against starvation stress. Increased level of phenol oxidase and acid phosphatase was the sign of activation of intracellular phagocytic responses. Higher level of lipid peroxidation and total hydroperoxides signify the resultant oxidative stress in the starved specimen. Modulation of antioxidant enzymes indicates adaptive behaviour in the tissue against oxyradicals. Decreased levels of protein content, ascorbic acid and reduced glutathione shows their utilization during starvation stress. **Conclusion:** The data revealed that starvation modulates immunological responses, oxidative stress and hemocytic nuclear fragmentation as a result of cellular damage and thus adversely affect overall fitness and quantitative traits of tasar silkworm.

Key words: *Antheraea mylitta*, starvation, hemocyte, oxidative stress, antioxidants, tasar silkworms

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Corresponding Author: R.K. Tiwari, Department of Zoology, Kashi Naresh Government Post Graduate College, 221304 Gyanpur, S.R.N. Bhadohi, India

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Food is the most vital component just as temperature, moisture and oxygen for the continuity of consistent life processes. Substantial nutritional and energy demands are linked with immune activation and its continued competence. Change in food availability intervenes the normal metabolic activities and fitness of living organisms[1-3]. Some information of starvation impact on insect development[4-8] and cocoon characteristics[9-12] is already available. Kehl and Fischer[13] reported that larval starvation reduces responsiveness to feeding stimuli and does not affect feeding preferences in a butterfly. Reisenman *et al.*[14] stated the effects of starvation on the olfactory responses of the blood-sucking bug *Rhodnius prolixus*. However, there appears to be no unanimity on the results of their impact and correlation with quantitative traits and immunological responses.

Oxidative metabolism of cells results in continuous production of Reactive Oxygen Species (ROS) from univalent reduction of O_2 that can damage most cellular components leading to cell death[3,15]. To protect against this unfavourable condition, insects possess a suit of antioxidant defence systems, composed of both enzymatic as well as non-enzymatic molecules. There appears to be a need, therefore, to obtain the detail information on immunological and oxidative responses of the starvation stress in tasar silkworm (*A. mylitta*). The major enzymes involved in the antioxidant defence process were superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione-S-transferase (GST). SOD converts superoxide radicals ($O_2^{\bullet-}$) to H_2O_2, which is reduced to water and molecular oxygen by CAT or is neutralised by GPX, that catalyses the reduction of H_2O_2 to water and organic peroxide to alcohol using GSH as a source of reducing equivalent. GR regenerates reduced glutathione (GSH) from oxidized glutathione (GSSG), which is a scavenger of ROS as well as a substrate for the other enzymes. GST conjugates xenobiotics with GSH for excretion. The non-enzymatic component consists of small organic molecules such as GSH and vitamin C[16-19].

Generally, rearing of the tropical tasar silkworm, *A. mylitta* carried out under outdoor condition on their food plants. During deficiency of food substantial nutritional and energetic demands of this silkworm may hamper. This could lead to variation in silk characteristics such as shell richness, denier, reeled silk length, cocoon weight and silk ratio etc. Although, *A. mylitta* is economically very important insect to produce a unique type of tasar silk, the impact of starvation stress on immunological and oxidative stress responses has not been studied much. The aim of the present work was, therefore, to assess the impact of starvation stress on immunological and oxidative stress responses of tasar silkworm *A. mylitta*. In this context, several immunological parameters of hemocytes as immune-markers i.e., Total Hemocyte Counts (THC), Differential Hemocyte Count (DHC), fat body lipid peroxidation (LPX), total hydroperoxides, the antioxidants CAT, GST, ASA, GSH, phenoloxidase (PO) and acid phosphatase (ACP) activities have been investigated.

MATERIALS AND METHODS

Study area: The present exploratory nature of work was conducted during July, 2014-September, 2019 during the rearing season of tasar silkworm at Central Tasar Research and Training Institute (Ministry of Textiles, Govt of India) Ranchi Jharkhand India. The hemocyte related experiment was conducted in the RK Tiwari's Laboratory located at K.N. Govt. PG College (MGKV University), Gyanpur, SRN Bhadohi, India.

Stock maintenance of *A. mylitta*: Stock of tropical tasar silkworm, *A. mylitta* Drury Daba ecorace was maintained in outdoor condition in field during second crop (September-November) rearing season. Larvae used in the present study were fed on the leaves of its primary food plant Asan, *Terminalia tomentosa* Wight&Arn in the experimental farm of Central Tasar Research and Training Institute Ranchi.

Starvation stress: The Vth instar 0 day old larvae were segregated and fed on Asan plant *T. tomentosa.* Generally, after completion of 5-6 days feeding, V instar larvae attain the ecdysial weight. Kumar *et al.*[20] reported that first peak of PTTH and ecdysone begins on 5th day of Vth instar *A. mylitta* larvae and this peak is necessary for larval-pupal conversion. Therefore, to provide starvation stress, 6-7 day old V instar larvae were starved by transferring them on branches of *T. tomentosa* devoid of leaves by covering the plant by net. Separate control lot was also maintained and larvae were fed normally on *T. tomentosa* leaves. Both the lots were left to remain on the host plant (*T. tomentosa*) till they spun the cocoons.

Morphological and developmental study: Morphological differences in control and starved insects such as deformity in larvae and pupae, variation in duration of larval-pupal transformation, commencement of cocoon formation, pupa formation duration and deformities in adult molting were observed.

Haemolymph smears and slide preparation: Haemolymph samples were obtained from live pupae by puncturing the anterior region by using sterilized needle in 70% ethanol. Ten microlitre haemolymph was drawn on a slide and mixed well with anti-coagulant, a thin uniform film was prepared by pulling the edge of an inclined slide backward. The film was air-dried at room temperature and stained. The methods of smear formation were similar to those applied earlier with slight modification[21-23].

Total Hemocyte Count (THC): For THC, the hemolymph was drawn from pupae into a thoma blood-cell pipette up to its 0.5 mark and diluted up to the 11th mark with Tauber-Yeager's fluid[23-24].

Differential Hemocyte Count (DHC): Counting of at least 200 cells of different categories selected from random areas of stained smears of 30 insects from control and starved insect were made independently. To see the nuclear fragmentation, DAPI stain was used. The methods of hemocyte categorization and DHC count were similar to those applied earlier with minor alteration[23].

Microscopy: Light and phase contrast microscopes were used to study the THC, DHC, different cell morphology and contour. Hemocytes were categorized based on basis of their morphology and staining reaction as observed under light and phase contrast microscopes. The Giemsa stained hemolymph smear slides were utilized for the study of hemocyte morphology. DAPI stain was used to see the impact on nuclear fragmentation by using florescent microscopes.

Haemolymph and fat body sample preparation: The hemolymph drawn from control and starved insects in a 1.5 mL eppendorf tube by mixing 0.01% phenyl-thiourea was utilized for biochemical estimation. Phenyl-thiourea was not added in samples which were collected for PO activity. The fat body was dissected out and washed thoroughly in ice-chilled phosphate buffer (50 mM, pH 7.0) to remove haemolymph contamination. Five replications of fat body homogenates were prepared from both male and female pupae. Tissue samples (fat body) were homogenized in ice-cold buffer (50 mM phosphate buffer, pH 7.0 and 1 mM DTT). Homogenization was carried out in an ice-chilled Teflon homogenizer and centrifuged at 8000 xg for 15 min at 4°C. The supernatant was used for biochemical analysis.

Estimation of protein: The fat body and hemolymph protein content in control and starved insects were estimated according to Bradford's assay method[25] and absorbance was measured at 595 nm. Concentration of the protein samples was determined from a standard curve drawn using bovine serum albumin.

Estimation of phenoloxidase activity: The PO activity was recorded in the form of dopachrome production, a product of the oxidation of L-dopa by the enzyme PO. The reaction mixture contains 850 µL phosphate buffer (100mM, pH 7.4), 100 µL (10mM, L-dopa) and 50 µL haemolymph. Absorbance at 490 nm was measured with a spectrophotometer. Absorbance was measured immediately after adding the L-dopa and again after 45 min. The PO activity was recorded as A490/min/mL haemolymph.

Acid phosphatase activity profile: Different samples of hemolymph and fat body of control and starved insects were subjected to ACP activity which was determined according to Arif *et al.*[26]. The p-nitrophenol was used for the preparation of a standard curve. The activity of the enzyme was articulated as nmol of p-nitrophenol released/h/mg protein.

Determination of lipid peroxidation: The extent of lipid peroxidation was assayed by assaying malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acids. Hydro peroxides were determined by the reaction with thio-barbituric acid (TBA) as described by Ohkawa *et al.*[27]. The absorbance was read at 532 nm after removal of any precipitating material by centrifugation. The amount of thiobarbituric acid reactive substances (TBARS) formed was calculated by using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as nmol TBARS formed/mg protein.

Estimation of total hydroperoxide: Hydroperoxides were determined spectrophotometrically according to the method of ferrous oxidation with xylenol orange (FOX1)[28]. Hydroperoxides oxidize ferrous to ferric ions selectively in dilute acid and the resultant ferric ions can be determined by using ferric sensitive dyes as an indirect measure of hydroperoxide concentration. Xylenol orange binds ferric ions with high selectivity to produce a coloured (blue-purple) complex. The absorbance was read at 560 nm after removal of any flocculated material by centrifugation at 4000 xg for 10 min. The signal was read against H_2O_2 standard curve and expressed as nmol H_2O_2/mg proteins.

Estimation of catalase activity: Catalase activity was determined according to Aebi[29] by following initial rate of decomposition of 20 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) and 100 µL of the assayed enzyme extract in a total volume of 3 mL. Catalase activity was estimated by the decrease in absorbance of H_2O_2 at 240 nm and was expressed as nkat/mg protein (1 katal = 1 mol sec^{-1}).

Estimation of glutathione-S-transferase activity: Glutathione-S-transferase activity was measured according to Habig *et al.*[30] using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. Assay mixture contained 2.7 mL of 100 mM phosphate buffer (pH 6.9), 30 mM GSH, 15 mM CDNB and 0.1 mL of sample (100-150 µg protein). The change in absorbance was recorded at 340 nm and enzyme activity was expressed as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}.

Estimation of reduced glutathione: Samples were precipitated in 5% (w/v) TCA in an ice bath and centrifuged at 5000 xg for 10 min. The deproteinised supernatant was used for the estimation of GSH. The content was estimated using Ellman's (DTNB) reagent[31]. Aliquots of supernatant (0.5 mL) were mixed with 0.6 mM DTNB and incubated for 30 min at room temperature in dark. The sample was finally centrifuged at 2,000 xg for 10 min at room temperature and the absorbance of the supernatant was measured at 412 nm. GSH was taken as standard and expressed as µmol GSH/mg protein.

Estimation of ascorbic acid: Ascorbic acid content was determined according to the method of Mitsui and Ohta[32]. The reaction mixture contained 2% sodium molybdate, 0.15 N H_2SO_4, 1.5 mM Na_2HPO_4 and 0.5 mL of sample. The mixture was boiled at 90°C for 45 min and then centrifuged at 1,000 xg for 15 min. The absorbance of the supernatant was measured at 660 nm. ASA was taken as standard and results were expressed as µg ASA/mg protein.

Cocoon assessment: Based on cocoon shell richness, uniformity in cocoon shape, double cocoon, flimsy cocoon, melted cocoon and morphologically different types of cocoons were separated. The cocoon and shell weight were calculated by using standard protocol with slight modification.

Statistical analysis: Results were expressed as Mean+Standard Deviation (SD). Difference among the control and treatment means of experiment was analyzed by Student's t-test. Differences were considered statistically significant when $p<0.05$.

RESULTS

Starvation of late V instar larvae caused a great deal of variation on growth, development, immunological responses and enzyme profile of *A. mylitta*. Surprisingly in starved group, reduction in larval duration 4-6 days has been observed which leads to early pupation when compared to fed larvae (Fig.1a). Almost 10% of the larvae died before pupation and 15% could

Table 1: Impact of starvation on rearing performance of *A. mylitta*

	Cocoon characters		
	Cocoon weight (g)	Pupal weight (g)	Shell weight (g)
Male			
Control	10.80±0.57	09.28±0.05	1.52±0.21
Starved	8.33±0.56*	07.17±0.07*	1.16±0.17*
Female			
Control	14.24±1.3	12.37±1.13	1.88±0.34
Starved	10.55±2.65	09.53±2.58	1.02±0.46*

Data are expressed as Mean±SEM (n = 30). *Indicate significant difference at $p<0.05$

Table 2: Changes in THC and DHC in late V instar male larvae of *A. mylitta* normal and starved conditions

		Different hemocyte types (%) (mean±SD)						
Insect group	THC (No. of cells/mm^3 of haemolymph) (mean±SD)	PRs	PLs	GRs	SPs	ADs	OEs	VEs
Male								
Control	19758±226	6.7±0.3	32.1±2.6	29.2±1.0	10.3±1.2	14.4±1.1	5.6±0.5	1.7±0.2
Starved	13145±324**	13.1±0.7***	21.7±1.4*	17.9±1.6***	22.6±2.3**	16.3±0.9	5.4±0.1	3.0±0.4*
Female								
Control	19936±332	4.8±0.4	29.8±2.1	24.7±1.2	24.5±1.2	12.5±0.9	3.7±0.4	Not found
Starved	14241±348**	12.7±1.0**	29.2±2.1	33.3±2.7*	10.3±1.9**	7.6±1.3*	4.4±0.4	2.5±0.4*

*,**,***Indicate significant difference at $p<0.05$, $p<0.01$ and $p<0.001$, respectively, THC: Total hemocyte count, PRs: Prohaemocyte, PLs: Plasmatocytes, GRs: Granulocytes SPs: Spherulocytes, ADs: Adipohaemocytes, OEs: Oenocytoids, VEs: Verrnicytes

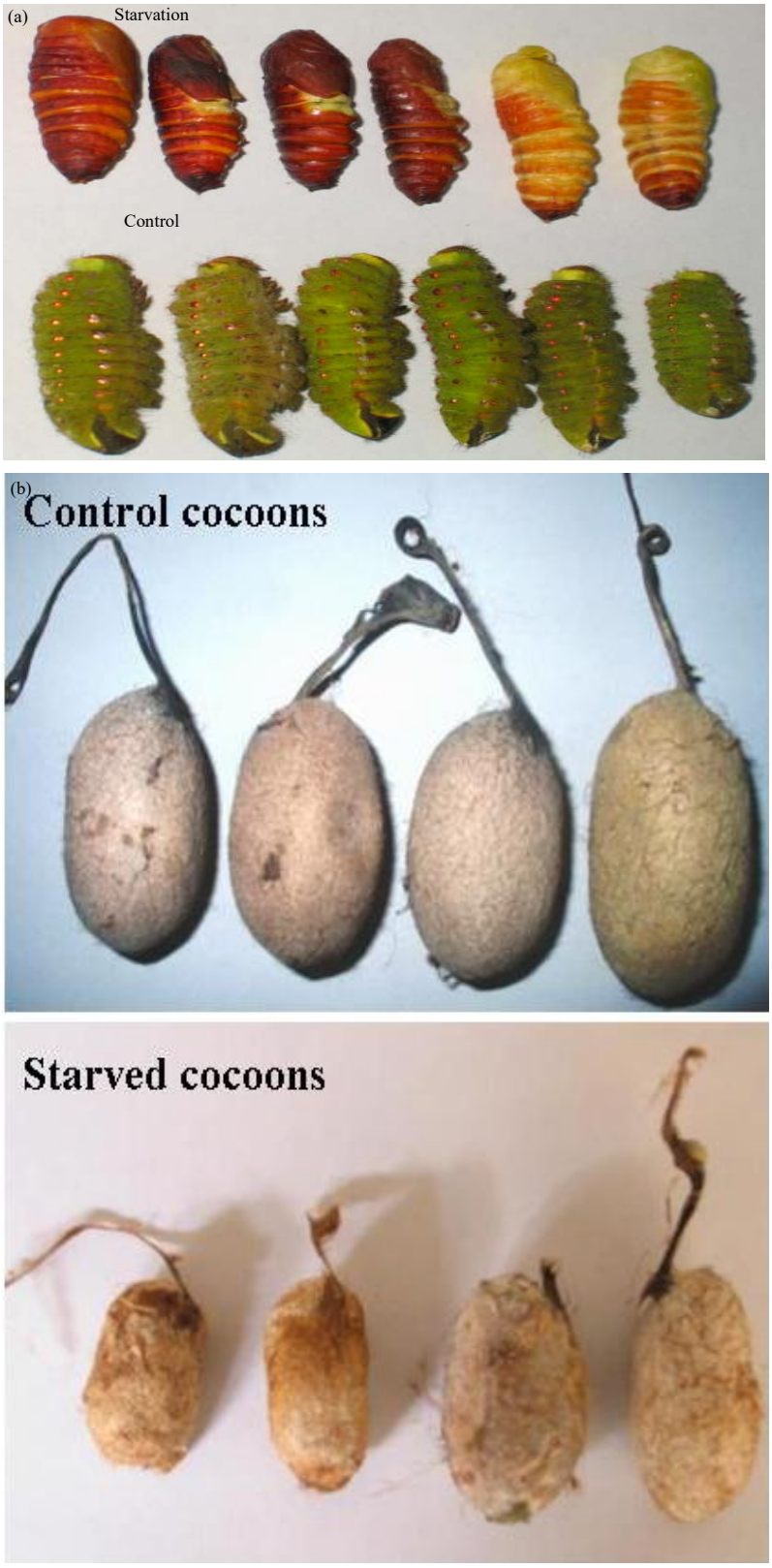

Fig.1(a-b): (a) Early pupae formation in starved insects in comparison to control (b) Shape and size of cocoons produced by control and starved larvae

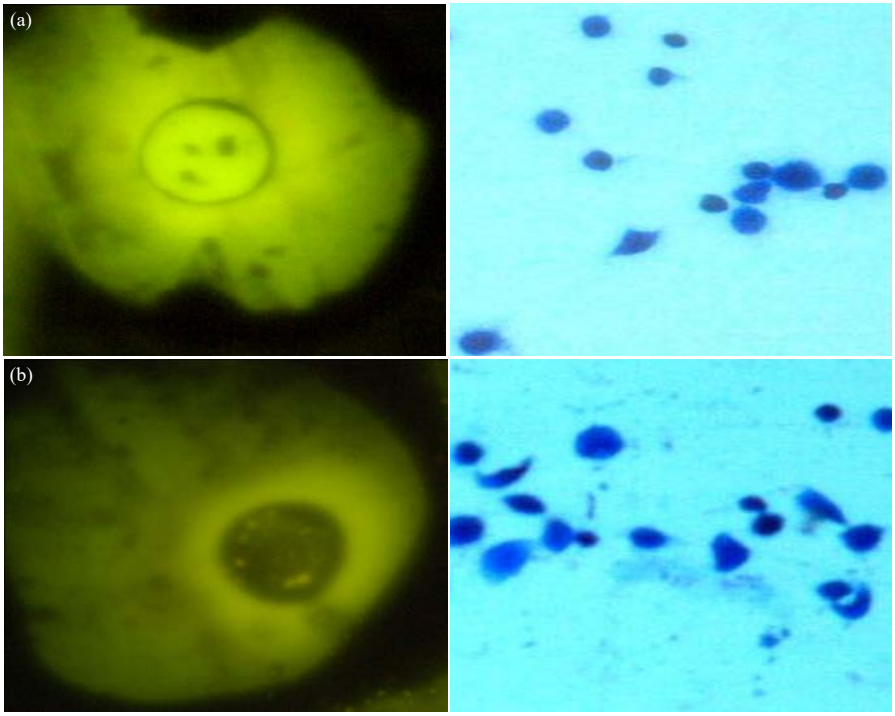

Fig. 2(a-b): Nuclear fragmentation and disintegration of hemocytes in starved larvae (a) Control and (b) Starved

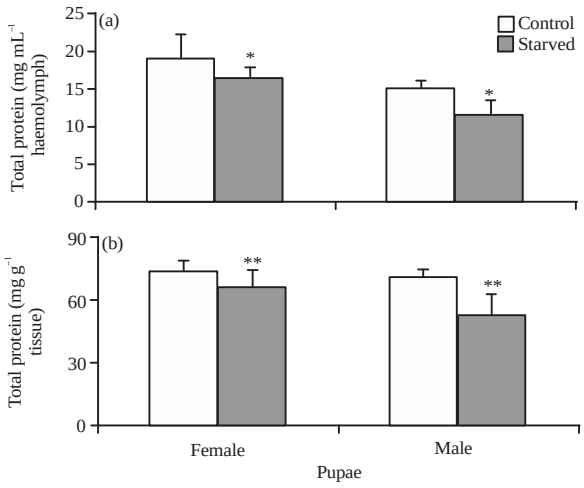

Fig. 3(a-b): Total protein concentration in (a) Haemolymph and (b) fat body, of control and starved male and female pupae

Data are expressed as Mean±SEM (n = 5), *,**Indicate significant difference at p<0.05 and p<0.01, respectively

form flimsy/deformed cocoons (Fig.1b) and these deformed cocoons yielded adult with crumpled wings. Starvation had negative impact on cocoon weight and shell weight also (Table 1).

Effect on cellular immune responses: Changes in total and differential hemocyte counts were observed under starved stress condition. Starvation stress badly affected the different categories of hemocytes (haemolymph cells), their profile, structure and counts accompanied with morphological changes in them. Haemolymph immune cells especially plasmatocytes (PLs) and granulocytes (GRs) got adversely affected and showed nuclear fragmentation and disintegration leading to 20% reduction in their count (Fig. 2). The 2-3 fold increase in prohemocyte (PRs) number was also observed in starved insects (Table 2). Although, THC and DHC vary in male and female, their response to starvation was apparent. Significant decrease in THC was observed in starved insects as compared to controls. Surprisingly, the VEs were not found in control females (Table 2).

Effect on biochemical indices: Protein levels in response to starvation stress are shown in Fig. 3a,b. The results showed significant decrease in protein of haemolymph and fat body of both male and female pupae, level of significance being higher in case of male specimen. The ACP activity was markedly elevated in the fat body of male and female pupae exposed to starvation in relation to control (Fig. 4a,b). Similar to fat body, ACP activity in haemolymph showed significant increase up to 2.55 in male than female pupae of starved larvae (Fig. 4a,b, p<0.05). Increased PO activity

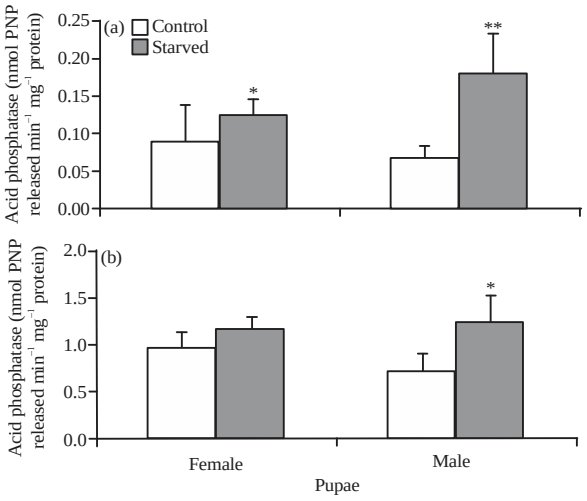

Fig. 4(a-b): Acid phosphatase activities in (a) haemolymph and (b) fat body of control and starved male and female pupae

Data are expressed as Mean±SEM (n = 5), *,**Indicate significant difference at p<0.05 and p<0.01 respectively

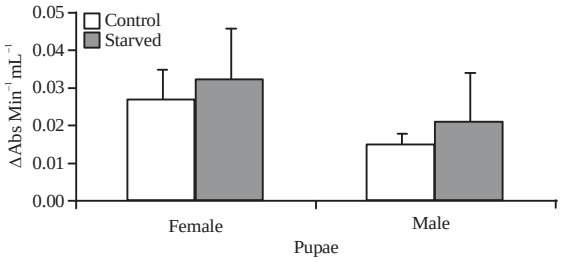

Fig. 5: Phenoloxidase activities in haemolymph of control and starved male and female pupae

Data are expressed as Mean±SEM (n = 5), *,**Indicate significant difference at p<0.05 and p<0.01, respectively

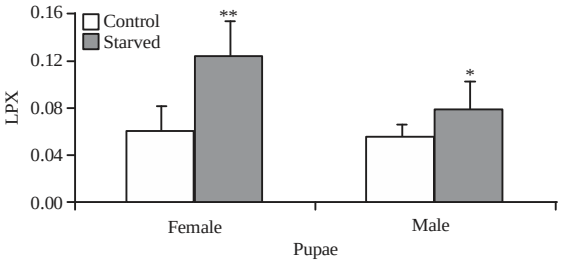

Fig. 6: Lipid peroxidation (nmol TBARS formed/mg protein) level in fat body of control and starved male and female pupae

Data are expressed as Mean±SEM (n = 5), *,**Indicate significant difference at p<0.05 and p<0.01, respectively

although detected in starved haemolymph (Fig. 5) the results were not statistically significant, probably due to high variance. During starvation stress, fat body of both male and female pupae showed significant elevation in LPX level as compared to control (Fig. 6, p<0.05). Likewise, total hydroperoxide (hydrogen peroxide and other hydro peroxides) level was also significantly high in fat bodies of starved pupae (Fig. 7a, p<0.05). However, CAT activity was significantly reduced in starved pupae of both males and females in comparison with control groups (Fig. 7b, p<0.05). A high GST activity in fat body was recorded in male and female starved pupae (Fig. 7c). GSH content was significantly reduced in fat body of male starved pupa in comparison to that of control but no significant increase was recorded in female specimen (Fig. 7d, p>0.05). A significant lower ASA content was observed in both male and female starved pupae than controls (Fig. 7e, p<0.05).

Effect on cocoon character: Starvation caused significant reduction (p<0.05) in cocoon weight and shell weight. The cocoon traits and its quantitative characters of tasar silkworm reared in two different conditions are presented in Table 1. The shell content expressed as the ratio between shell weight and cocoon weight was also significantly reduced (p<0.05) in starved insects. Inferior quality cocoons were produced by starved insects with less shell richness and intactness. Deformity in cocoon formation was also observed in starved insects.

DISCUSSION

Lack of food can limit the ability of organisms to sustain the stressful conditions. While changes in food availability, intervene the normal metabolic activity and fitness of living beings. During outdoor rearing *A. mylitta* experiences periods of starvation or limited food availability at moulting stage. Impact of starvation on various parameters of late V instar larvae of *A. mylitta* has been discussed as under.

Changes in THC, DHC and cell contour in test insects can be attributed to the response of haemolymph cells to starvation stress. Elevation in prohaemocyte (PRs) numbers ascribed as immediate response of insect to maintain the total number of cells. Since, THC has also decreased in starved insects; the PRs being stem cell nature[33,34], elevation in their count could have occurred to compensate the number of cells in profile. It is reported that the PRs profile is regulated by the ecdysone in insects[35,36]. Similar to present findings, the decrease in number of immune cells i.e., PLs and GRs and their morphological changes, nuclear fragmentation and cellular disintegration have also been observed in starved insects[8].

Starvation studies give indication of the energy utilized by silkworm and provide clues to the biochemical pathways involved under these conditions. Significantly low level of protein observed in fat body and haemolymph of starved pupae (Fig. 3a,b, $p<0.05$) suggest that organisms compensate starvation using endogenous reserve proteins. The starvation stress was more prominent in male than in female pupae. The life cycle of female *A. mylitta* being longer than male the higher protein content in females suggest that protein stocks in females could be more or its utilisation be slow during starvation. The relative importance of metabolic reserves and their order of utilization vary among species reviewed in Hervant *et al.*[37]. Similar to the present findings low proteins are also reported in starved animals[38].

Marked variation in ACP activity in control and starved insects may be the indication of tissue degradation. Since fat body in insect works analogous to liver of higher organisms, the ACP activity in fat body may indicate the starvation stress. It is reported that the action of this enzyme in many tissues is to cleave a waste product called pyrophosphate and effectively convert it into a usable phosphate. Elevation in ACP activity in starved insects fat body male and female and similar variation in haemolymph is due to starvation induced damage to cells. Changes in ACP activity in fat body during insect development and its relation with ecdysteroids has been observed by several researchers[26,39]. The 2-3 fold increase in the count of PRs in starved insects also indicates its relation with ecdysone titre present in hemolymph. It also corroborates the finding of Tiwari and Shukla[35] and Pandey *et al.*[36] who have reported that the PR's profile is regulated by the ecdysone. Present result indicates that the PRs and ACP can be utilised as a marker for ecdysone which is very important hormone to decide the health condition of insect.

It is reported that PO is the key enzyme in the biochemical cascade of β-sclerotization, quinone tanning and melanin biosynthesis, which occupies a major role in insect development and immunity. In the present study, phenoloxidase (PO) activity was enhanced during starved condition (Fig. 5), which indicates activation of melanisation. PO is copper proteins of wide occurrence in nature which catalyze the aerobic oxidation of certain phenolic substrates to quinones which are autoxidised to dark brown pigments generally known as melanins. It is assumed that elevation of PO under starved condition is due to breakdown of hemolymph cells. Decrease in level of PO activity has also been observed by Lin *et al.*[40] after starvation stress. It is known that the process of melanization in insects is accompanied by formation of the potentially toxic ROS including semiquinone radicals[41], hydrogen peroxide[42] and superoxide anion[43]. Such cytotoxic ROS can lead to an uncontrolled increase of lipid peroxidation.

Lipid peroxidation is a well known mechanism of cellular injury in both vertebrates and invertebrates and acts as an indicator of oxidative stress in cells and tissues[44]. An enhanced level of TBARS in the fat body of pupae was observed in response to starvation stress (Fig. 6). Our results clearly demonstrate that *A. mylitta* starvation stress is accompanied by lipid peroxidation and other responses to oxidative stress, similar to rainbow trout, *Oncorhynchus mykiss*[45], sea bream, *S. aurata*[46]. It has been reported that food deprivation induces the production of oxyradicals[46-48], which could possibly increase the rate of ROS formation and resulting in oxidative stress. Except total hydroperoxides H_2O_2 and other water soluble hydroperoxides[28], no measurement of O_{2^-}, $OH^{\bullet}$ and other ROS markers were made in this study. According to these results the higher levels of total hydroperoxides were observed in starving groups as compared to control in present study (Fig. 7a). H_2O_2 can alter cell physiology through the formation of $OH^{\bullet}$ by Fenton reaction[49]. This situation could be responsible for the high LPX level observed in pupae exposed to starvation stress, which could have affected the quantitative traits of silkworm (Table 1).

Organisms are equipped with a cascade of enzymes to counteract ROS produced either during normal metabolism or due to exposure to various stresses[50]. In this cascade, SOD is the first to dismutate $O_2^{\bullet -}$ to H_2O_2 which is further scavenged by CAT and GPX. Significant decrease in CAT activity in fat body of the pupae due to starvation indicates its utilisation due to increased production of H_2O_2 or $^{\bullet}OH$ by Haber-Weiss reaction leading to increased TBARS content in the tissues. Similar to this finding, lower CAT activity was also observed in fasting fish *Sparus aurata*[47]. Further, increase in antioxidant enzyme levels such as GST in fat body may be an adaptive strategy by the insect to counteract oxidative stress generated as a consequence of starvation stress. GST catalyzes the conjugation of reduced GSH to nucleophilic xenobiotics or cellular components damaged by ROS[51]. Increased GST activities observed in fat body of starved groups (Fig. 7c) are similar to those reported by Bayir *et al.*[52] for branchial tissue of *Salmo trutta*. Increased GST activity may conjugate intermediate metabolites with GSH resulting in detoxification of endogenous molecules and thereby protecting the tissues from oxidative stress of starvation. Earlier studies also documented significant relationships between GST and LPX in testes of *A. mylitta* exposed to thermal stress[53].

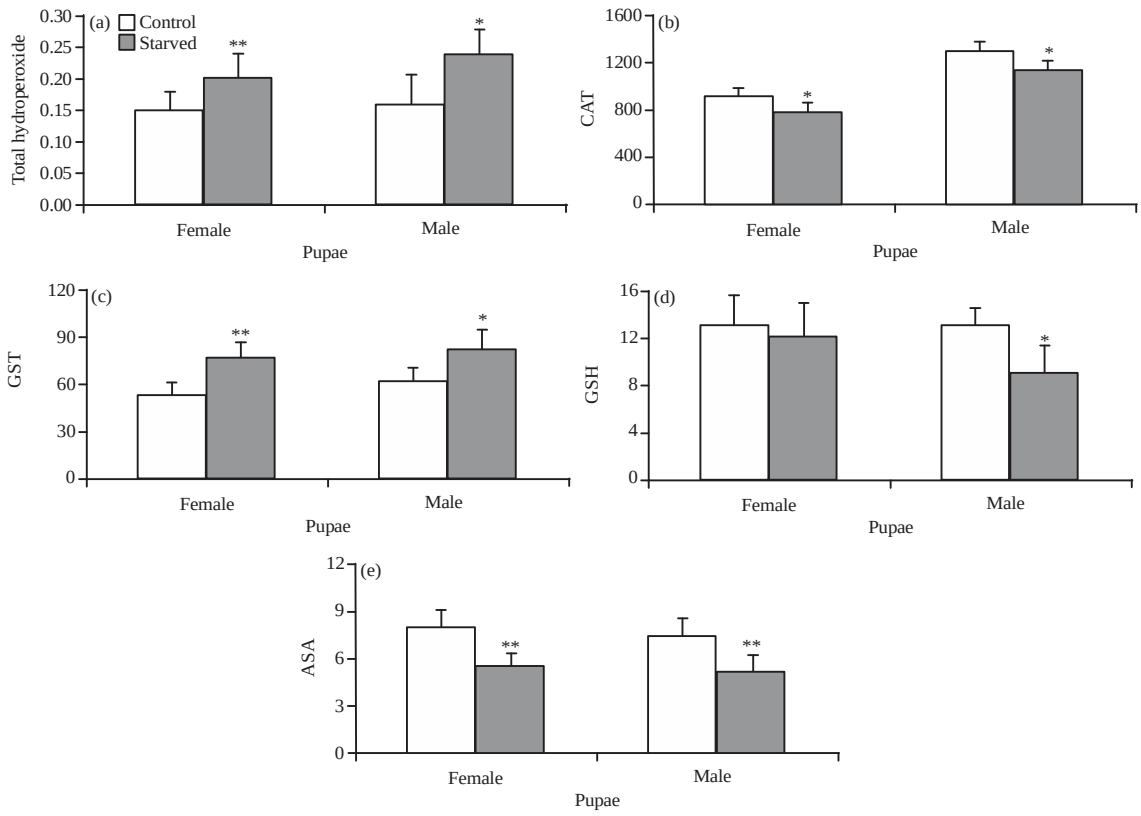

Fig. 7(a-e): (a) Total hydroperoxide (nmoL mg^{-1} protein), (b) Catalase (nkat mg^{-1} protein), (c) glutathione-S-transferase (nmol CDNB conjugate formed $min^{-1}mg^{-1}$ protein), (d) Reduced glutathione (µmol GSH mg^{-1} protein) and (e) Ascorbic acid (µg ASA mg^{-1} protein) in fat body of control and starved male and female pupae
Data are expressed as Mean±SEM (n = 5), *,**Indicate significant difference at $p<0.05$ and $p<0.01$, respectively

Depletion of GSH level was also observed in fat body of starved pupae in present study (Fig. 7d). An increase in GST activity coupled with decreased GSH values, suggest higher utilization of GSH as a response to combat prooxidant release during starvation. This indicates utilization of the antioxidant more than its synthesis under starvation stress. Similar response was also detected in rat liver and carcinoma cells during starvation condition[54,55].

The ASA is known to directly scavenge ROS[50]. In the present study low ASA content observed in fat body of starved pupa (Fig. 7e) may be indicative of either dietary deficiency or its utilization in response to elevated ROS obtained through starvation stress. Such condition may arise due to decreased availability of GSH in which ASA plays an important role in reduction of dehydroascorbate to ascorbate[56]. Further studies may be needed to understand the mechanism involved in use of ASA during starvation.

It is apparent that starvation induced changes in cocoon characteristics of *A. mylitta* are a process of energy management for survival under starvation stress. These observations corroborate the findings of Chaudhuri *et al.*[4], Samson *et al.*[9], Satish and Govindan[10], Janarthanan *et al.*[11] and Bhaskar *et al.*[12]. Similarly, Li *et al.*[2] investigated the impact of starvation and hormones on DNA synthesis in silk gland cells of the silkworm, *Bombyx mori* and found that levels of 4E-BP phosphorylation in the silk glands were also reduced by starvation and *in vivo* treatment with 20HE. It is reported by Rovenko *et al.*[3] that high sucrose consumption promotes obesity whereas its starvation (low consumption) induces oxidative stress in *Drosophila melanogaster* which is reflected by higher levels of oxidized lipids and proteins accompanied by increased superoxide dismutase activity. Interestingly, Li *et al.*[2] reported that DNA synthesis could be inhibited by starvation and re-activated by re-feeding and therefore appears to be dependent on nutrition. DNA synthesis was suppressed by *in vivo* treatment with 20-hydroxyecdysone (20E). However, there was no effect on DNA synthesis by *in vitro* 20E treatment or by either *in vivo* or *in vitro* juvenile hormone treatment. Knapp and Uhnava[1] found that total fecundity was more strongly affected by

feeding in comparison to the structural body size of females. Since larvae were starved after attaining ecdysial weight and threshold age, their reduction in larval period under starved condition in *A. mylitta* is due to realization of unavailability of food by neurosecretory cells (NSCs) of the brain. These brain cells could increase the rate of synthesis and release of prothoracicotropic hormone (PTTH) from brain and ecdysone from prothoracic glands (PTG). The result substantiates the findings of Kumar *et al.*[20] where it is reported that two major peaks of PTTH and ecdysone were noticed during fifth instar larval development of *A. mylitta*. Hormone required for normal growth and development of *A. mylitta* could be altered (or probably increase in ecdysone titre) under starved condition leading to reduction in duration of larval stage and developmental failure. Growth and development of insect is regulated by 20-hydroxyecdysone (20-HE) and Juvenile Hormone (JH). It is reported that JH suppresses the growth and development of imaginal tissues after ecdysis in starved *Manduca sexta* larvae[57]. Changes in biochemical profile cellular immune responses, shell richness and cocoon characteristics may be taken as an indication of immediate physiological response and management of energy by the present insect to reduce the silk yield to sustain existence in starved condition.

CONCLUSION

Present study was an attempt to correlate starvation v/s hemocyte population, cocoon shell richness, biochemical parameters and contour of hemocytes. The observations presented a first comprehensive report on total haemocyte count, differential counts, oxidative stress and antioxidant defence system in pupae of tasar silkworm *A. mylitta*. The results indicate a significant induction of PRs, SPs, LPX and total hydroperoxides and reduction of THC and CAT following starvation stress. These may adversely affects the quantitative traits of *A. mylitta* pupa. Further, low level of GSH and ASA may be interpreted as possible protection by non-enzymatic antioxidants, which could be sensitive to ROS generated under starvation stress.

SIGNIFICANCE STATEMENT

Tasar silkworm is an economically important insect producing the tasar silk which is known for its unique quality and lustre. Late fifth instar larvae are the voracious feeder which sometimes gets starved during rearing. Therefore, its impact needs to be explored significantly. This study quantified the impact of starvation on immuno-oxidative stress and cocoon characteristics of tasar silkworm *A. mylitta* successfully. This crucial information will form the basis for planning the rearing experiments. In addition, the present study will also help the researchers to uncover the critical areas linked to overall impact of starvation for further exploration.

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