Oxidative Damaged Products, Level of Hydrogen Peroxide, and Antioxidant Protection in Diapausing Pupa of Tasar Silk Worm, *Antheraea mylitta*: A Comparative Study in Two Voltine Groups

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ABSTRACT: The present study demonstrates tissue-specific (hemolymph and fat body) and inter-voltine [bivoltine (BV) and trivoltine (TV)] differences in oxidatively damaged products, H_2O_2 content, and the relative level of antioxidant protection in the diapausing pupae of *Antheraea mylitta*. Results suggest that fat body (FB) of both the voltine groups has oxidative predominance, as evident from the high value of lipid peroxidation and H_2O_2 content, despite better enzymatic defenses in comparison to hemolymph (HL). This may be attributed to the higher metabolic rate of the tissue concerned, concomitant with high lipid content and abundance of polyunsaturated fatty acids (PUFA). Nondetectable catalase activity in the pupal hemolymph of both strains apparently suggests an additional mechanism for H_2O_2 metabolism in the tissue. Inter-voltine comparison of the oxidative stress indices and antioxidant defense potential revealed that the TV group has a higher oxidative burden, lower activities for the antioxidant enzymes, and compensatory nonenzymatic protection from reduced glutathione and ascorbic acid.

KEYWORDS: pupal diapause, voltinism, oxidative stress, antioxidants, hemolymph, fat body, *Antheraea mylitta*

CITATION: Sahoo et al. Oxidative Damaged Products, Level of Hydrogen Peroxide, and Antioxidant Protection in Diapausing Pupa of Tasar Silk Worm, *Antheraea mylitta*: A Comparative Study in Two Voltine Groups. *International Journal of Insect Science* 2015:7 11–17 doi:10.4137/IJIS.S21326.

RECEIVED: October 29, 2014. **RESUBMITTED:** January 5, 2015. **ACCEPTED FOR PUBLICATION:** January 23, 2015.

ACADEMIC EDITOR: Helen Hull-Sanders, former Editor in Chief

TYPE: Original Research

FUNDING: Authors disclose no funding sources.

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

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Introduction

Diapause is a state of physiological dormancy characterized by diminished oxygen consumption.[1] Insects in diapause alter their developmental program, modify the composition of cellular membranes, increase the amount of storage lipids, and regulate the expression of some stress-responsive genes.[2–4] It is an important aspect of insect development and physiologically an adaptive mechanism against low-temperature injury and desiccation. Understanding of insect diapause has tremendous biological significance in population modeling, pest management, aging, and the invention of novel defense components, cryoprotectants, and regulators of cell cycle.[3]

The tropical tasar silkworm, *Antheraea mylitta* (Lepidoptera–Saturniidae), is a species of sericulture importance and has the second largest capacity for silk production among all the silk-spinning insects.[5] It exhibits well-defined voltinism, mostly BV (bivoltine: two generation per year) and TV (trivoltine: three generation per year), and completes its life cycle with an impressive pupal diapause. The duration of pupal phase (from 0-day pupa to adult eclosion) in the BV group is more (200–210 days) than that of the TV group, which is 150–160 days. Though several comprehensive reviews provide a good deal of information on insect diapause,[2–4,6–8] our knowledge on insect diapause in relation to cellular redox status, ie, the status of pro-oxidants and antioxidants, is comparatively small,[9,10] and particularly for the tropical tasar silkworm, it is virtually nil.

Reactive oxygen species (ROS), such as the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$) act as pro-oxidants and cause oxidative damage to important biomolecules of the cell when they cross their physiological limits. Antioxidant defense plays an important role in battling against ROS and thus protects the cell from the ROS-mediated oxidative assault. Studies on the expression and activity of antioxidant enzymes in insects suggest that the cellular redox state regulates numerous physiological processes and may impair the survival, growth, development, fecundity, fertility, and adult life-span.[11–15] The antioxidant defense system is especially important in wild and semidomesticated phytophagus insects because they are exposed to ROS-promoting environments such as UV radiations, environmental pollutants, pathogenic agents, and, sometimes, pro-oxidative plant allelochemicals.[16–18] Bindu et al have suggested that UV exposure is a major source of pro-oxidative insult encountered by the tropical tasar silkworm.[19] ROS, particularly

H_2O_2, which regulates intracellular redox potential, exhibits dual roles in the cellular environment. By its oxidative properties, it acts as a pro-oxidant, and through its function as a second messenger it modulates signaling pathways.[20] H_2O_2-mediated lipid peroxidation (LPX) has devastating consequences in the insect cells especially during the developmental process because lipids have many important physiological functions.[21] On the other hand, the endogenous ROS level of an individual is well related to cellular metabolism, O_2 consumption, mitochondrial respiration, development, and differentiation.[22,23] Since insect diapause is characterized by a regulated metabolic rate concomitant with reduced O_2 consumption, a low level of endogenous ROS is expected. However, extra storage of lipids and changes in membrane architecture during diapause push the insect toward elevated oxidative assault, particularly LPX.[24]

Though studies on this species in relation to diapause and voltinism suggest a marked inter-voltine difference in oxygen consumption and in some biological traits of the diapausing pupa,[25] no information is available on the level of ROS, ROS-mediated damage products, and antioxidant protection of this economically important insect species with reference to their strain and voltinism. Therefore, the present set of experiments is designed to analyze tissue-specific oxidative-damage products, the level of H_2O_2, and relative level of antioxidant protection in two important tissues, namely the hemolymph (HL) and fat body (FB), of diapausing pupa of two voltine groups (BV and TV), which differ in their rate of O_2 consumption and pupal life-span.

Materials and Methods

Chemicals. Thiobarbituric acid (TBA), bovine serum albumin (BSA), Sephadex G-25, and 5,5′ dithiobis 2-nitrobenzoic acid (DTNB) were purchased from Sigma Chemical Co., USA. *N′N′*-bis-acrylamide and *NNN′*N′-tetramethylethylenediamine (TEMED) were purchased from Merk-Schudart, and reduced glutathione, ascorbic acid, horse radish peroxidase, hydrogen peroxide, 2,4-dinitrophenylhydrazine (DNPH), guanidine hydrochloride, acrylamide, sodium dodecyl sulfate (SDS), ferric chloride, potassium ferricyanide, and ammonium persulfate were obtained from SISCO Research Laboratory, India. Digitonin was procured from Hi-media Ltd., India. All other chemicals used were of analytical grade.

Insects. Cocoons containing the pupae of the tasar silk worm (*A. mylitta*) from bivoltine (BV) and trivoltine (TV) strains were collected from identical host plants (*Terminalia arjuna*) of the same age group maintained by the State Government Sericulture Field, Baripada, Orissa, India. After collection, they were maintained in the laboratory under natural photoperiod (12 hour light: 12 hour dark) and room temperature (26 ± 2°C). Pupae on PED 60 (pre-emergence day 60, ie, 60 days prior to the probable date of adult eclosion) were taken and removed after opening the shell (silken case) of the cocoon, and apparently healthy pupae were used in the experiment.

Tissue preparation. Prior to collection of tissues, pupae were carefully brushed to remove contaminating particles. Hemolymph was collected by puncturing their head in an ice-chilled Eppendorf tube coated with 0.03% phenylthiourea to prevent melanization. Hemolymph samples were centrifuged at 3,000 *g* for 10 minutes at 4°C to get rid of hemocytes. The supernatant fraction was stored at −40°C for further experiments. FBs of the pupae were dissected out, washed in ice-cold physiological saline (0.67%), and weighed in monopan balance after pat-drying on filter paper. A 10% (w/v) homogenate of the FB in 50 mM phosphate buffer, pH 7.4, was prepared with the help of a glass–Teflon mechanical homogenizer on ice. The homogenates were centrifuged at 10,000 *g* for 20 minutes at 4°C to get the post-mitochondrial supernatant (PMS). Endogenous H_2O_2 content and catalase (CAT) activity were measured in the PMS immediately after centrifugation. The remaining supernatant was kept at −40°C for further analysis of other related parameters.

Lipid peroxidation. The level of LPX was assayed by monitoring the formation of malondialdehyde (MDA), according to the method of Ohkawa et al.[26] In brief, a 10% (w/v) homogenate of the FB was prepared in 1.15% KCl and centrifuged at 1,000 *g* for 10 minutes at 4°C to remove cell debris. Suitably diluted HL fraction and 1,000 *g* supernatant of FB were used for the estimation of MDA. All samples were treated with 0.02% butylated hydroxytoluene to prevent endogenous oxidation. The amount of MDA formed was calculated from the extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$,[27] and was expressed as nanomoles MDA per milligram of protein.

Protein carbonyl. The protein carbonyl (PC) content of the respective tissue fractions was determined using the method of Levine et al.[28] This assay was used for determining the content of oxidatively modified proteins through the detection of carbonyl groups. These carbonyl groups react with 2,4-dinitrophenyl hydrazine (DNPH) to form hydrazone derivatives whose concentration was determined spectrophotometrically at 366 nm using a complementary blank. The carbonyl content was calculated from extinction coefficient of $22{,}000\ M^{-1}\ cm^{-1}$ and expressed as nmol PC/mg protein.

Hydrogen peroxide. The H_2O_2 content in HL and post-mitochondrial fraction of FB was determined spectrophotometrically using horse radish peroxidase and H_2O_2 as standard, according to the method of Pick and Keisari.[29]

Superoxide dismutase activity assay. For the estimation of total superoxide dismutase (SOD), 0.4 mL of supernatant containing approximately 10–15 mg of protein was passed through a 2-mL column of Sephadex G-25, and the elute was used for the activity assay of SOD according to the method of Das et al.[30]

Catalase activity assay, native PAGE, and activity staining. The post-mitochondrial supernatant was used directly for the assay of catalase (CAT) activity following the decrease in absorbance of H_2O_2 at 240 nm.[31] To detect

the CAT activity in gel, samples were run in a 10% polyacrylamide gel without SDS according to the method of Laemmli.[32] The CAT activity was stained by the method of Woodbury et al.[33] The gel after the run was washed three times with distilled water and incubated for 5 minutes in 0.01% H_2O_2 with hand-shaking at room temperature. The gel was washed three times with distilled water and stained in the staining solution (final concentration 1% ferric chloride and potassium ferricyanide each). When transparent bands (exhibiting CAT activity) were visualized after 2 minutes, 1% HCl was added to stop the reaction, and the gel was washed properly with distilled water.

Protein content. Protein content of the samples for the assay of oxidative stress indices and antioxidant enzymes was estimated according to the method of Lowry et al[34] using bovine serum albumin as standard.

Glutathione (GSH) content. The supernatant from the FB homogenate and the hemolymph fractions isolated earlier were incubated with 5% (w/v) sulfosalicylic acid in ice and centrifuged at 1000 *g* for 10 minutes. GSH was analyzed using DTNB by the method of Ellman.[35]

Ascorbic acid content. Hemolymph and FB samples were incubated with 5% (w/v) TCA in ice and centrifuged at 1000 *g* for 10 min. The supernatant was taken for the estimation of ASA using Folin Phenol reagent and ascorbic acid as standard by the method of Jagota and Dani.[36]

Statistical analysis. All data are reported as means ± SEM for n = 5 samples (in duplicate) with one pupa per sample. Data were analyzed using the *t*-test with standard computer analysis software (Microsoft excel, 2003) and considered statistically significant when at least $P < 0.05$ between the compared groups as well as between the two tissues of an identical group.

Results

Oxidative damaged products (LPX and PC). Comparison of oxidative stress indices, ie, the level of LPX (malondialdehyde content, MDA) and PC content in the pupal tissues of *A. mylitta* is shown in Figure 1A and B, respectively. The LPX level showed a higher value for the FB than hemolymph in both the voltine groups with a magnitude of 508% and 732% higher MDA content in FB than hemolymph in BV and TV groups, respectively (Fig. 1A). Inter-voltine difference in the LPX level in either of the tissues was not significant. However, a clear inter-voltine difference in the PC content was observed in the hemolymph of the pupa: ie, around 560% higher PC value was observed in the hemolymph of the TV group than that of BV group (Fig. 1B). Though tissue-specific variation in PC content is not significant in the BV pupa, TVs exhibited remarkable tissue specificity for the PC value.

H_2O_2 content. A comparatively high level of H_2O_2 was observed in the FB of both pupae (Fig. 2). The H_2O_2 content in the hemolymph exhibited a significant inter-voltine difference, which was 168% more in the TV pupae. Tissue-specific variation of H_2O_2 was also observed in the BV pupae.

Antioxidant enzymes. In both the voltine groups, the total SOD activity was found to be much higher in the FB than in the hemolymph, ie, 240% and 87% higher value for BV and TV, respectively (Fig. 3A). Though the total SOD activity of the hemolymph was almost identical in both the groups, FB of the TV group showed a lower SOD activity (44%) than the BV pupa. CAT activity was below the level of detection in the hemolymph of either voltine group. Nevertheless, CAT activity was detected in the FB of both groups without any significant inter-voltine difference (Fig. 3B). To ascertain the nondetectablity of CAT in the hemolymph, in addition to spectrophotometric analysis we also tried native PAGE staining. Though a fairly good amount of activity was observed in the FB (transparent band L_3 and L_4), no CAT activity was detected in hemolymph (L_1 and L_2) (Fig. 4). Live pupae were also treated with 10 mM H_2O_2 for possible induction of CAT in the pupal hemolymph. This exogenous administration of H_2O_2 failed to induce CAT activity in the tissue concerned, and even exogenous H_2O_2 treatment could

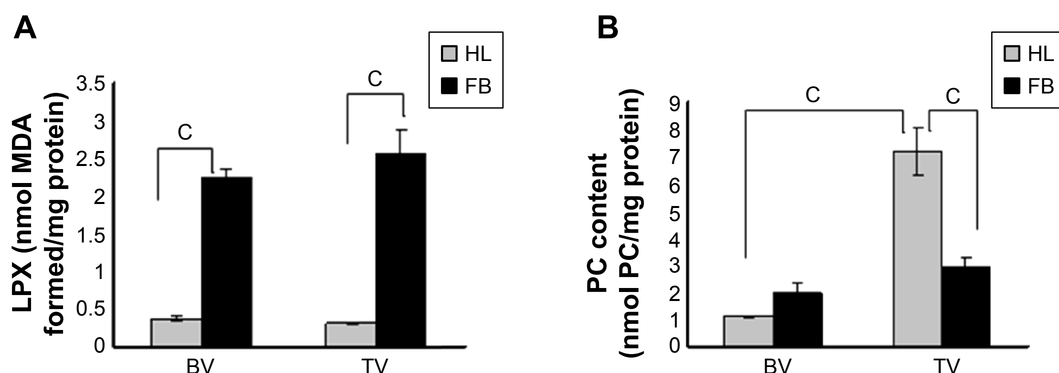

Figure 1. (**A**) Endogenous LPX level (nmol MDA/mg protein) in the hemolymph (HL) and fat body (FB) of BV and TV pupae. Values are mean ± SEM of five animals. Bars sharing superscript of different letters differ significantly ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$). (**B**) Protein carbonyl content (nmol PC/mg protein) in the hemolymph (HL) and fat body (FB) of BV and TV pupae. Values are mean ± SEM of five animals. Bars sharing superscript of different letters differ significantly ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$).

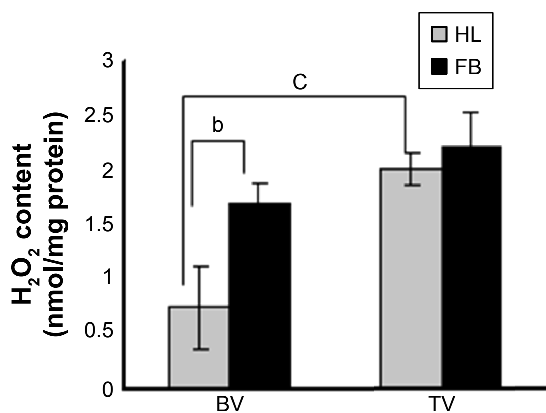

Figure 2. H_2O_2 content (nmol/mg protein) in the hemolymph (HL) and fat body (FB) of BV and TV pupae. Values are mean ± SEM of five animals. Bars sharing superscript of different letters differ significantly ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$).

not alter the endogenous H_2O_2 level in the hemolymph (data not shown).

Nonenzymatic antioxidants. The GSH content of the pupal FB was much higher than the corresponding value of hemolymph in both the voltine groups. However, it was observed that inter-voltine difference of GSH in either of the tissues was not significant (Fig. 5A). ASA also exhibited a similar trend like GSH in its distribution with reference to tissue specificity and voltinism (Fig. 5B).

Discussion

Results of the present study indicate that the oxidatively damaged products, H_2O_2 content, and the level of antioxidant protection in the pupa of *A. mylitta* are tissue-specific and exhibit inter-voltine differences. Endogenous LPX level (MDA content) is more in case of FB than hemolymph in both BV and TV pupae. FB is a lipid-rich organ homologous to vertebrate liver and crustacean hepatopancreas. It is also one of the important sites for intermediary metabolism[37] and expected to generate more ROS and ROS-generating enzymes. Moreover, FB is also an important organ for lipogenesis in insects having abundant PUFA.[38] Besides, changes in the cell membrane composition during diapause, ie, toward more unsaturation in the fatty acyl chain,[39] render them more susceptible to oxidative attack. Therefore, the abundance of PUFA, the degree of unsaturation, and elevated ROS production in FB may be the reasons for the high tissue peroxidation products in comparison to hemolymph. The H_2O_2 content observed in the FB is higher than in the hemolymph, which also strongly supports the above fact. On the other hand, lower MDA level in the hemolymph of the pupa than in the FB may be attributed to the low lipid content and comparatively low metabolic rate of the tissue. The present findings are in good agreement with those of earlier reports, which also indicate that antioxidant defense and oxidative stress were more pronounced in the tissues of insects having high metabolic activities, such as malpighian tubules, FB, and hind gut, in comparison to hemolymph.[12]

The PC content exhibited a differential value with reference to tissue and voltinism. The higher PC value in the hemolymph of TV pupae in comparison to that in the hemolymph of BV group suggested a strong inter-voltine difference. The higher tissue PC content in the hemolymph of TV pupae can be correlated with their high oxygen consumption[25] in comparison to BV pupae. Since the rate of oxygen consumption is directly related to ROS release,[22] higher tissue oxidation products in the tissues of TV pupae than BV is logical. PC content is a general measure of oxidative damage[40] and is considered as a key indicator of oxidative stress.[16] It is further noticed that, in the hemolymph of TV group, the antioxidant status such as the activity of total SOD and the contents of GSH and ASA were less with a relatively high H_2O_2 level. Thus, the poor antioxidant defense along with high H_2O_2 content may be the reason for the high PC content in the hemolymph of the TV group. Krishnan et al[16] have observed a significant increase in the PC level in the tissues of *Spodoptera littoralis*

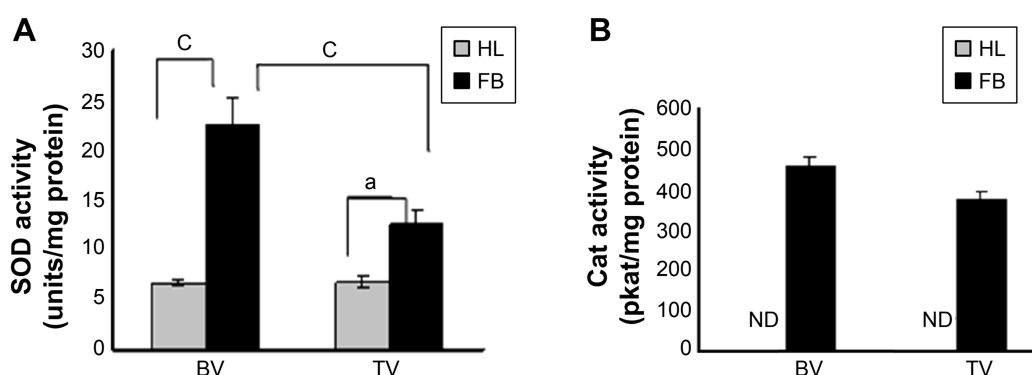

Figure 3. (**A**) SOD activity (units/mg protein) in the hemolymph (HL) and fat body (FB) of BV and TV pupae. Values are mean ± SEM of five animals. Bars sharing superscript of different letters differ significantly ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$). (**B**) Catalase activity (pkat/mg protein) in the fat body (FB) of BV and TV pupae. Values are mean ± SEM of five animals.
Abbreviation: ND, Not detected.

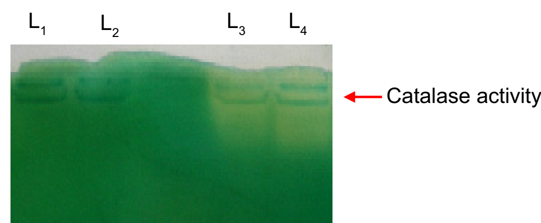

Figure 4. Representative PAGE photograph of catalase activity (*n* = 3) in the hemolymph and fat body of BV and TV pupae. L_1 – trivoltine hemolymph, L_2 – bivoltine hemolymph, L_3 – trivoltine FB, and L_4 – bivoltine FB.

in response to oxidative stress. Wang et al[41] also noticed that the overexpression of Mn SOD in virus-infected insect cells resulted in a significant reduction in oxidative protein damage. These findings further support the role of antioxidants in the protection of protein from oxidative attack.

Inter-voltine difference in the hemolymph and FB H_2O_2 content being higher in TVs may be a consequence of more O_2 consumption of this group. Comparatively high metabolic rate in the pupal FB can also be linked to the elevated H_2O_2 level in this group. Substantially high level of H_2O_2 in both the tissues of TV pupae might also have some physiological relevance with reference to their pupal diapause termination and adult eclosion. Several experimental evidences support that H_2O_2 is a potent modulator that can influence physiological and developmental processes in many organisms.[20,42] H_2O_2 has been reported to regulate the development of quiescent *Artemia* cysts and their diapause termination.[42] Robbins et al[42] further suggested that H_2O_2 may be generated within *Artemia* cysts during development under the influence of external cues and environmental signals. Diapausing pupa of *A. mylitta* slowly moves from quiescence to active phase of development prior to termination of diapause. Zhao and Shi[20] also have opined that H_2O_2 is involved in the termination of diapause in the mulberry silkworm *Bombyx mori* through cell signal transduction. The work of Fujiwara et al[43] strongly suggests the involvement of extracellular signal-related kinase (ERK) and mitogen-activated protein kinase (MAPK) in the termination of diapause in *B. mori*. Thus, it can be hypothesized that TV group with higher H_2O_2 level possibly activates the signal transduction pathways and thereby enhance the rate of morphogenesis and results in adult eclosion in shorter duration (150–160 days) than BV pupae (200–210 days).

Total SOD activity in the hemolymph of both BV and TV pupae was found to be much lower than in FB and no catalase activity was observed in the hemolymph of either pupal groups. Lower SOD activity in the hemolymph than in FB and nondetectable CAT activity in the hemolymph may suggest either attenuated production of $O_2^{\cdot -}$ in the hemolymph due to lower rate of metabolism than FB or the dominance of nonenzymatic antioxidative defense potential. Hemolymph is known to generate ROS;[44,45] however, it possesses very low activity for most of the antioxidant enzymes such as SOD, CAT, and GST.[12] Perhaps trehalose, uric acid, and ascorbic acid are the primary antioxidants in insect hemolymph.[12] It has also been observed that many insects during their wintering (diapause) accumulate glycerol and ethylene glycol at a very high concentration as a part of antioxidant defense. Nondetectable CAT activity in the pupal hemolymph of this insect species observed in our study is consistent with the earlier findings of Ahmad.[12] Recently, we also observed that inhibition of CAT by aminotriazole had no effect on the endogenous H_2O_2 level in the pupal fat body of this insect.[46] This indicated the presence of an efficient mechanism for the neutralization of H_2O_2 other than CAT. Several studies have shown that an ascorbate recycling enzymatic system and peroxiredoxins are involved in the elimination of H_2O_2 in insects.[9,47–49]

GSH and ASA contents were more in FB than in hemolymph of both voltine groups. GSH is an important antioxidant component of most aerobic cells[50] and also acts as a cellular redox buffer.[51] GSH depletion has been shown to intensify LPX and predispose cells to oxidative damage.[52] Summers and

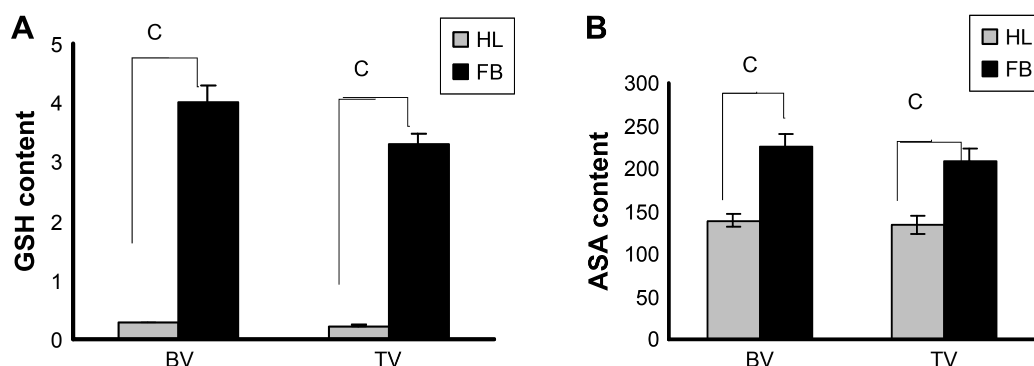

Figure 5. (**A**) GSH content of hemolymph (HL) (µmol/ml) and fat body (FB) (µmol/g tissue wet wt.) in BV and TV pupae. Values are mean ± SEM of five animals. Bars sharing superscript of different letters differ significantly ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$). (**B**) ASA content of hemolymph (HL) (µg/ml) and fat body (FB) (µg/g tissue wet wt.) in BV and TV pupae. Values are mean ± SEM of five animals. Bars sharing superscript of different letters differ significantly ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$).

Felton[53] observed that ascorbic acid is an effective antioxidant in protecting *Helicoverpa zea* tissues against phenolic prooxidants. Therefore, higher GSH and ASA contents observed in the FB than hemolymph might be an adaptive antioxidant response for better protection from oxidative assault. Jovanovic-Galovic et al[9] also observed a higher ASA content in the pupal tissues of the European corn borer in their mid-diapause stage. GSH and ASA are important for their protective role against ROS and form a powerful redox couple to neutralize a variety of free radicals in biological systems.[53]

Taken together, findings of this study suggest that pupal FB of both voltine groups is under higher oxidative threat concomitant with better antioxidant protection. It is further observed that between the two voltine groups, the TV pupae experience more oxidative assault with simultaneous induction of nonenzymatic antioxidants (GSH and ASA), probably as an adaptive cellular response to elevated ROS level due to the higher oxygen consumption. Nondetectable CAT activity along with a substantial level of H_2O_2 in the pupal hemolymph suggests an important physiological function of H_2O_2 in pupal development. This study also sheds light on our understanding of diapause in relation to the pro-oxidant–antioxidant balance in the tissue environment (cellular redox balance). Better understanding of insect diapause will help us to develop specific strategies for the management of insect diapause, ie, prevention of untimely entry into diapause and synchronization of adult ecolsion by minimizing erratic emergence. So, synchronized diapause will improve the reproductive potential of this insect, better hatchability, and increased survival of the larvae. Collectively, all these events will make a good contribution to the field of sericulture and silk production.

Acknowledgments

We are thankful to Prof. S.K. Dutta, former Head, Department of Zoology, North Orissa University, Baripada, India, and Prof. G.B.N. Chainy, former Head, Department of Zoology and Biotechnology, Utkal University, Bhubaneswar, India, for the provision of Laboratory facilities to complete the work. We thank Mr. Jajati Rout, State Silk Board (Baripada) for providing live cocoons. Financial assistance from the Department of Biotechnology, Govt. of India, to the P.G. Department of Biotechnology, Utkal University, is gratefully acknowledged.

Author Contributions

Conceived and designed the experiments: JD and LS. Analyzed the data: AS and JD. Wrote the first draft of the manuscript: AS. Contributed to the writing of the manuscript: AS, JD and LS. Agree with manuscript results and conclusions: AS, JD and LS. Jointly developed the structure and arguments for the paper: AS, JD and LS. Made critical revisions and approved final version: AS, JD and LS. All authors reviewed and approved of the final manuscript.

Supplementary Data

Supplementary figure 1. Interaction between pro-oxidants (ROS) and antioxidants in cellular environment. Pro-oxidants: superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH^-$). Antioxidants: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), oxidized glutathione (GSSG), ascorbic acid (ASA), dehydroascorbic acid (DHA). Oxidized damaged product: Lipid peroxidation (LPX), protein carbonyl (PC).

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