Sahoo et al. BMC Zoology (2018) 3:1
DOI 10.1186/s40850-018-0027-4

BMC Zoology

RESEARCH ARTICLE Open Access

Low H_2O_2 and enhanced oxidative resistance in the diapause-destined pupa of silkworm, *Antheraea mylitta* (Lepidoptera:Saturniidae) suggest their possible involvement in dormancy and lifespan extension

Alpana Sahoo[1], Abinash Dutta[2], Jagneshwar Dandapat[2*] and Luna Samanta[3]

Abstract

Background: Insect diapause is associated with physiological dormancy, extension of life span and an altered developmental programme. Reactive oxygen species, oxidative damaged products and antioxidants are being increasingly linked to such processes in insects. Tropical tasar silkworm, *Antheraea mylitta* Drury (Lepidoptera: Saturniidae), has an impressive pupal diapause and exhibits a distinct pattern of development in nondiapausing (NDP) pupa compared to that of diapausing (DP) pupa. In this study, we have analysed the amount of hydrogen peroxide (H_2O_2) and oxidative damaged products along with the levels of antioxidant protection in the hemolymph (HL) and fat body (FB) tissues of developing pupa of *A. mylitta* from DP and NDP generations having identical chronological age but distinct physiological status.

Results: Signature of oxidative predominance was clearly marked in the tissues of diapause-destined pupa as evident from a higher level of lipid peroxidation and protein carbonyl content, despite a lower level of H_2O_2 in the said group. Specific activity of catalase, exclusively observed in the FB was significantly higher in diapause-destined pupae. Total superoxide dismutase activity was found to be higher in both tissue types of diapause-destined pupae compared to their NDP counterpart. In contrast, pupae committed for diapause displayed less activity for glutathione S-transferase than the NDP group. Reduced glutathione exhibited tissue specificity being higher in the FB of NDP pupae.

Conclusions: Findings of this study suggest that low H_2O_2 pool with a strategic antioxidant protection is associated with induction of dormancy and enhanced oxidative resistance during an extended lifespan.

Keywords: Antioxidants, Fat body, Hemolymph, Hydrogen peroxide, Oxidative resistance, Pupal dormancy, Longevity

* Correspondence: jdandapat.nou@gmail.com; jd.biotech@utkalunivesity.ac.in
[2]Department of Biotechnology, Utkal University, Bhubaneswar, Odisha 751004, India
Full list of author information is available at the end of the article

Background

Insect diapause, a pause and an alteration in developmental programme, is a biological process characterized by diminished oxygen consumption and lower metabolic rate [1]. It is an incredibly interesting phenomenon in developmental biology and aging in which insects suddenly alter their O_2 consumption, become dormant and extend their life span [2–4]. The tropical tasar silkworm, *Antheraea mylitta* Drury (Lepidoptera:Saturniidae), an insect of sericulture importance, exhibits well-defined pupal diapause. *A. mylitta* has distinct developmental stages in its life cycle i.e. egg, larva, pupa (inside cocoon) and the moth stage. Eggs are hatched into young worms called larvae. The process of larval development has five stages also known as instars (I^{st}, II^{nd}, III^{rd}, IV^{th} and V^{th} instar). The larval stage is the only feeding stage in its entire life cycle. Larva, in its final (V^{th}) instar, attains full growth, stops feeding and spins a protective covering around itself popularly known as cocoon. It then changes into pupa, which exhibits facultative diapause [5] possibly to cope with the extremes of environment and shortage of food. *A. mylitta* exhibits three distinct patterns of life cycle with reference to the time duration of one generation, popularly referred to as voltinism. The voltine varities (uni-, bi- and tri-voltine i.e. one, two and three generations in a year) are conspicuous and distinct in relation to the duration of the pupal phase. In case of trivoltine strain, the pupae developing from larvae of the first two generations (1st and 2nd) are generally non-diapausing (NDP) in nature and have a comparatively short pupal period of 15–21 days. In contrast, the pupae developing from the larvae of 3rd generation are destined for diapause with an extended pupal life spanning around 160 days. It is pertinent to mention here that despite a differential duration of pupal period, individuals from both the generations achieve the same level of morphogenesis at the end of pupal development. Cascades of cellular events during pupal development are associated with the cellular redox balance, the balance between oxidants and antioxidants [6]. Reactive oxygen species (ROS) like superoxide radicals (O^{-}), hydroxyl radicals ($OH^{\cdot}$) and hydrogen peroxides (H_2O_2) are common oxidants and inflict oxidative damages on important biomolecules of the cell when they cross their physiological limits. Lipid peroxidation (LPx) and protein carbonyl (PC), two important oxidative damaged products, have been linked with increased ROS level and extension of life span in some insect model [7]. Under normal conditions, the presence of antioxidant defense system protects the cell from ROS-mediated oxidative assaults. The antioxidant defense system consists of an array of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) along with some low molecular weight components like reduced glutathione (GSH) and ascorbic acid (ASA) [8]. In insects, ROS, particularly H_2O_2, serve as a key regulatory molecule not only in various physiological processes, it also plays an important role in the regulation of cell differentiation, development and diapause [9, 10]. H_2O_2 exhibits a dual role in the cellular environment: by its oxidative properties it acts as a prooxidant and through its function acts as a second messenger. Though the involvement of ROS in the process of development has been well documented, most of the studies are limited to in vitro effects of ROS or based on the findings from cell culture [11] and far fewer experiments have been carried out on developing embryos or larvae. Albeit a few reports suggest the involvement of oxidants and antioxidants in the process of development and diapause of insects [12–14], we have little understanding of the pupal development of tropical tasar silkworm, particularly in relation to redox active components and diapause.

However, studies on this species during pupal development in DP and NDP generations suggest a marked difference in oxygen consumption [15, 16]. Oxygen utilization is associated with ROS production and oxidative process [17]. The present experiment is, therefore, designed to ascertain the level of H_2O_2, oxidative damaged products (LPx and PC) and relative levels of antioxidant protection in two important tissues i.e. hemolymph (HL) and fat body (FB) of *Antheraea mylitta* pupa from DP and NDP generations having identical chronological age (10 days old pupae) with different physiological states. In this study we hypothesize that the dynamics of ROS metabolism and status of antioxidants in response to differential O_2 consumption should be distinct and strategic in DP and NDP generations of pupae, which differ in their patterns of development and ageing.

Methods

Cocoons containing live pupae of tasar silk worm (*A. mylitta*) were collected from identical host plants (*Terminalia arjuna)* of the same age group maintained in the State government sericulture field located at Baripada (21° 56′ 6 N, 86° 43′ 17 E), Odisha, India. The pupae collected from the 2nd and 3rd generations served as NDP and DP groups, respectively. Ten days old pupae from both the generations were used in this experiment.

Tissue preparation

HL was collected from the healthy pupa in an ice chilled microcentrifuge tube coated with 0.03% phenylthiourea (PTU) to prevent melanization. Then the pupae were sacrificed, FB was collected and washed in physiological saline (0.67%), blotted dry and weighed in monopan balance. A 10% (*w*/*v*) homogenate was prepared in ice

cold 50 mM phosphate buffer, pH 7.4 containing 0.1 mM EDTA and a pinch of phenylmethane sulphonyl fluoride (PMSF) using glass teflon mechanical homogenizer. Fat body homogenates were centrifuged at 10,000 x g for 20 min at 4 °C and the post-mitochondrial supernatant was collected. The HL samples were centrifuged at 3000 x g for 10 min at 4 °C to get rid of hemocytes. Endogenous hydrogen peroxide (H_2O_2) content and catalase (CAT) activity were measured in the samples immediately after centrifugation. The remaining supernatant was kept at – 50 °C for further analyses. All measurements were done in triplicate.

Measurement of hydrogen peroxide (H_2O_2) content

The hydrogen peroxide 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 employed by Pick & Keisari [18]. In brief, in each tube, 1.7 ml of phosphate buffer (50 mM pH 7.4), 0.1 ml of phenol red solution and 50 μl of horse radish peroxidase (50 units) were taken and incubated for 5 min at room temperature. Next 0.1 ml sample was added to it followed by immediate addition of 50 μl of NaOH (1 N) to stop the reaction. H_2O_2 content was expressed as nmoles H_2O_2/mg protein.

Oxidative damaged products

Measurement of lipid peroxidation (LPx)

The level of LPx was determined by monitoring the formation of malondialdehyde (MDA) according to the method of Ohkawa et al. [19]. A 10% (*w*/*v*) homogenate of the FB was prepared in 1.15% KCl and centrifuged at 1000 x g for 10 min at 4 °C to remove cell debris. Suitably diluted HL fraction and 1000 x g supernatant of FB (having around 500 μg protein) 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 MDA i.e. 1.56×10^5 M^{-1} cm^{-1} [20] and was expressed as nmol MDA formed/ mg protein.

Measurement of protein carbonyl content (PC)

Protein carbonyl content was estimated according to the method adopted by Levine et al. [21]. This assay was used to determine the amount of oxidatively modified proteins through the detection of carbonyl groups that react with 2,4-dinitrophenyl hydrazine (DNPH) to form hydrazone derivatives, whose concentration was determined spectrophotometrically at 366 nm using the complementary blank. The carbonyl content was determined from the molar absorption coefficient of 22,000 M^{-1} cm^{-1} and expressed as nmol PC/mg protein.

Activities of antioxidant enzymes

Assay of superoxide dismutase (SOD) activity

For the measurement of total SOD activity, 0.4 ml of post-mitochondrial 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 according to the method of Das et al. [22] as described earlier [23]. SOD activity was expressed as units/mg protein.

Assay of catalase (CAT) activity

The post-mitochondrial supernatant was used directly for assay of catalase activity following the decrease in absorbance of H_2O_2 at 240 nm [24]. Calculation was done by taking the extinction coefficient of H_2O_2 as 43.6 M^{-1} cm^{-1}. Activity was expressed as pkat/mg protein (1pkat = 10^{-12} katal). One katal is defined as the amount of enzyme that transforms one mole of substrate per second.

Assay of glutathione S-transferase (GST) activity

The column-passed samples were used for the assay of glutathione S-transferase activity adopting the method of Habig & Jakoby [25]. In brief, 2.6 ml of 50 mM potassium phosphate buffer pH 6.5, 0.2 ml of 90 mM GSH and 0.1 ml of sample (160 μg of protein for FB and 200 μg for HL) were added to prepare the reaction mixture. The reaction was initiated by adding 0.1 ml of 30 mM CDNB (1-chloro-2,4-dinitrobenzene) to it. Absorbance was recorded at 340 nm at 1 min interval till 6 min. Calculation was done by taking the extinction coefficient of CDNB i.e. 9.61 mM^{-1} cm^{-1}. Activity was expressed as nmol CDNB conjugate formed/min/mg protein.

Estimation of reduced glutathione (GSH)

The supernatant from the FB homogenate and HL fractions isolated earlier were incubated with 5% (*w*/*v*) sulfosalicylic acid in ice and centrifuged at 1000 x g for 10 min. GSH content in the tissue samples was determined using DTNB (5,5′-Dithiobis-2-nitrobenzoic acid) according to the method of Ellman [26] and expressed as μmol/ml (for HL) or μmol/g tissue wet weight (for FB).

Estimation of ascorbic acid (ASA)

HL and FB samples were incubated with 5% (w/v) trichloroacetic acid (TCA) in ice and centrifuged at 1000 x g for 10 min. The supernatant was taken for the estimation of ASA using folin phenol reagent and ascorbic acid as standard according to the method adopted by Jagota & Dani [27] and ASA content of the tissue was expressed as μg/ml (for HL) or μg/g tissue wet weight (for FB).

Protein content

Protein content of the samples for the assay of oxidative damaged products and antioxidant enzymes was estimated according to the method of Lowry et al. [28] using bovine serum albumin as standard.

Statistical analysis

All data are reported as means ± S.E.M. for $n = 6$ samples. Data were analysed using t-test and considered as statistically significant when at least $p < 0.05$ between the DP and NDP generations as well as between two tissues of an identical group. Details of the statistical analysis have been included in the Additional file 1.

Results

H_2O_2 content and oxidative damaged products (Fig. 1a, b and c)

The level of H_2O_2 and oxidative damaged products like LPx (MDA content) and PC levels in HL and FB tissues of the pupae from DP and NDP generations of *A. mylitta* were found to be different in two different groups. FB tissues of the pupa from NDP generation have significantly higher level of H_2O_2 than those of their DP counterpart. In contrast, MDA and PC contents were found to be higher in both the tissue samples of the pupae from DP generation than the NDP sample.

Activities of antioxidant enzymes (Fig. 2a, b and c)

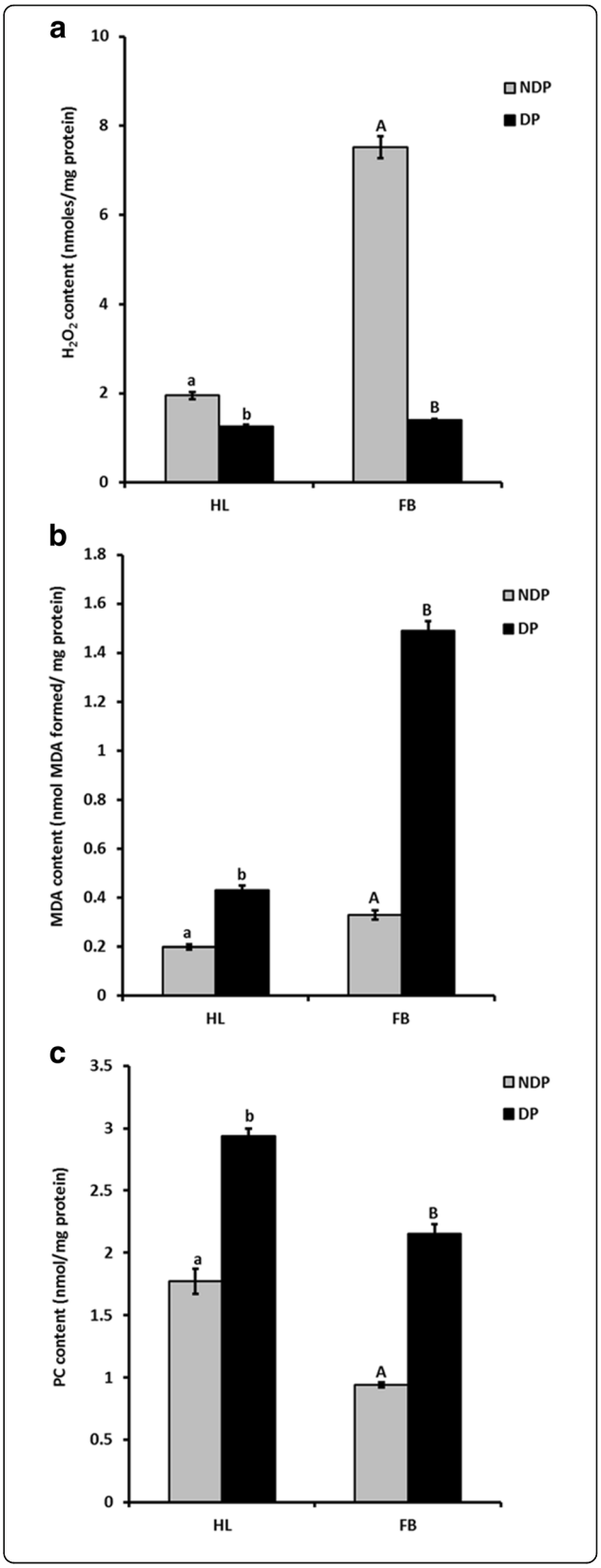
Higher specific activity of SOD was observed in both HL and FB tissues of the pupae from DP generation than those of the NDP group. Compared to NDP generation, CAT activity, exclusively detected in FB, was also significantly higher in the DP generation. It may be noted here that CAT activity was below the level of detection in HL under identical assay conditions. On the other hand, comparatively less GST activity was recorded in the pupal tissues (both HL and FB) of DP generation.

Non-enzymatic antioxidants (Fig. 3a and b)

GSH content exhibited tissue specificity. Though its level in HL was found to be equal in both the categories of pupae, for FB it was significantly more in NDP generation. Comparatively low level of ASA was recorded in both the tissues of the pupa destined for diapause than the NDP one.

Discussion

Findings of this study indicate that the metabolism of ROS, the level of oxidative damaged products and status of antioxidant protection are different in the pupae destined for diapause compared to NDP group. In case of DP generation, 10 days old pupae are approaching the preparatory phase of diapause. O_2 consumption is low, therefore, a low level of endogenous ROS is presumed



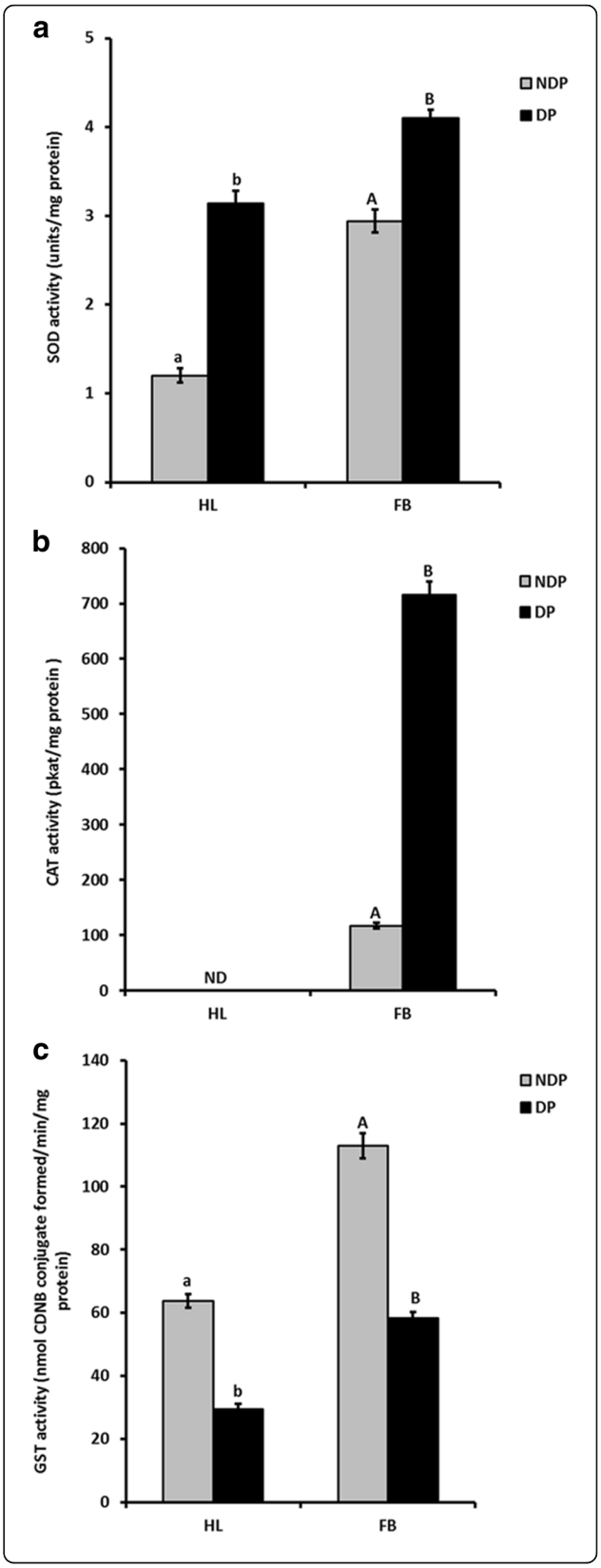


Fig. 1 a-c H_2O_2 content and oxidative damaged products. Level of H_2O_2 (**a**), MDA (**b**) and PC content (**c**) (nmol/mg protein) in the hemolymph (HL) and fat body (FB) of the pupae from nondiapausing (NDP) and diapausing (DP) generations. Bar represents mean ± S.E.M. ($n = 6$). Bars having different lower and upper case letters indicate significant difference in the measured parameters in HL and FB, respectively. Differences in the mean value have been considered statistically significant with a confidence interval of 95% and significant level of 5%

than in their NDP counterpart. Lower H_2O_2 content in diapause-destined pupal tissues compared with NDP pupa, as observed in the present study, substantiate our presumption and also corroborate the findings of Zhao et al. [9, 29] in *B. mori* and Jena et al. [30] in *A. mylitta*. Zhao and coworkers have documented low level of H_2O_2 and higher activity of catalase in the eggs of *B. mori* during the initiation of diapause. We have also observed a higher catalase activity in the FB of diapause destined pupae. Shen et al. [31] observed that exogenous treatment of H_2O_2 could prevent initiation of diapause in *B. mori.* In a similar study on *Artemia*, Robbins et al. [32] further confirmed that supplementation of H_2O_2 in the incubating medium (sea water) terminates diapause in *Artemia* cysts. Above observations and our present findings indicate that low H_2O_2 is implicated in the initiation of diapause. In contrast, 10 days old pupae of NDP generation are in an active phase of development, leading to the termination of pupal life within the next 5–10 days. NDP pupae have been reported to exhibit higher O_2 consumption than the DP counterpart [15, 16]. As O_2 utilization is associated with the level of ROS production [17], the higher amount of H_2O_2 in the tissues of NDP pupae, observed in this study, is obvious. Though a few reports indicate a reciprocal relation between higher respiratory activity and mitochondrial ROS release [33–36], further studies are required to establish the findings in this insect model with simultaneous measurement of O_2 consumption, metabolic rate and subcellular ROS generation in a single platform. Furthermore, we observed that despite a higher H_2O_2 level in the NDP pupae, the activities of SOD and CAT (exclusively observed in FB) were low in the same group. It is, therefore, assumed that the sequential actions of SOD and CAT may not be a major determining factor for H_2O_2 pool in this insect, as usually believed [37, 38]. Zhao and Shi [10] in their study on the metabolism of H_2O_2 in *B. mori,* indicated the existence of alternate mechanisms for the control of H_2O_2 in silkworm. Previously we also observed that CAT inhibition by aminotrizole in the pupal fat body of this insect did not result in the accumulation of H_2O_2 in the tissue concerned [39], which further substantiate the present findings.

Fig. 2 a-c Activities of antioxidant enzymes. Activities of SOD (**a**) (units/ mg protein), CAT (**b**) (pkat/mg protein) and GST (**c**) (nmol CDNB conjugate formed/min/mg protein) in the hemolymph (HL) and fat body (FB) of the pupae from nondiapausing (NDP) and diapausing (DP) generations. Bar represents mean ± S.E.M. ($n = 6$). Bars having different lower and upper case letters indicate significant difference in the measured parameters in HL and FB, respectively. ND:Not detected. Differences in the mean value have been considered statistically significant with a confidence interval of 95% and significant level of 5%

Irrespective of tissues, MDA content and PC values were higher in the pupae of DP generation in comparison to those in the NDP group. Changes in fatty acid compositions of membrane lipids, i.e., towards more unsaturation [40] and extra storage of tissue lipids for sustained use [41], as a preparatory change for diapause may be the reasons for the susceptibility of the tissues to LPx. High PC value in the diapause-destined pupae can be due to high levels of LPx, as interaction of LPx intermediates with side chains of amino acids is a determining factor for protein carbonylation [42]. We presume that more oxidative damaged proteins are associated with lifespan extension of the pupae committed for diapause as reported earlier [7, 43]. High SOD and CAT activities in the pupae destined for diapause may be a strategy for extended life span, as observed earlier by Orr & Sohal [44] in SOD and CAT overexpressed *Drosophila melanogaster*, by Sim & Denlinger [45] in the diapausing mosquito, *Culex pipiens* and also by Tasaki et al. [46] in the long lived queens of the termite, *Reticulitermes speratus*. Lower GST activity and GSH levels observed in the pupae of this group are in good agreement with the findings of Meng et al. [47] in *B. mori* eggs. Zhao et al. [48] also observed low GSH/GSSG ratio as an important factor for diapause determination in the bivoltine strain of *B. mori*. In contrast, higher GST activity and GSH content in NDP pupae observed in this study correspond to the findings of Jovanović-Galović et al. [6] with reference to the nondiapausing larvae and pupae of European corn borer, *Ostrinia nubilalis*. GSTs are involved in the process of detoxification in insects [49], regulate their metamorphosis [6] and metabolize lipid peroxides without exhibiting any activity towards H_2O_2 [50, 51]. Higher GST activity in the pupae of NDP generation, therefore, can be linked with the attenuation of oxidative damage as reported earlier [50, 52] and to early metamorphosis of the pupae concerned [6].

The contents of nonenzymatic antioxidants (GSH and ASA), higher in the NDP group compared to DP generation, seems to be a strategic antioxidant response for the protection of insect tissues from oxidative damage [53]. Higher levels of GSH may also be correlated with the rapid development of pupae in NDP generation, as GSH has been reported to augment cell division and differentiation [54]. Similarly lower level of ASA in the diapause-destined pupal tissues corroborates the findings of Dmochowska-Ślęzak et al. [14] in diapausing Red mason bees, *Osmia bicornis*.

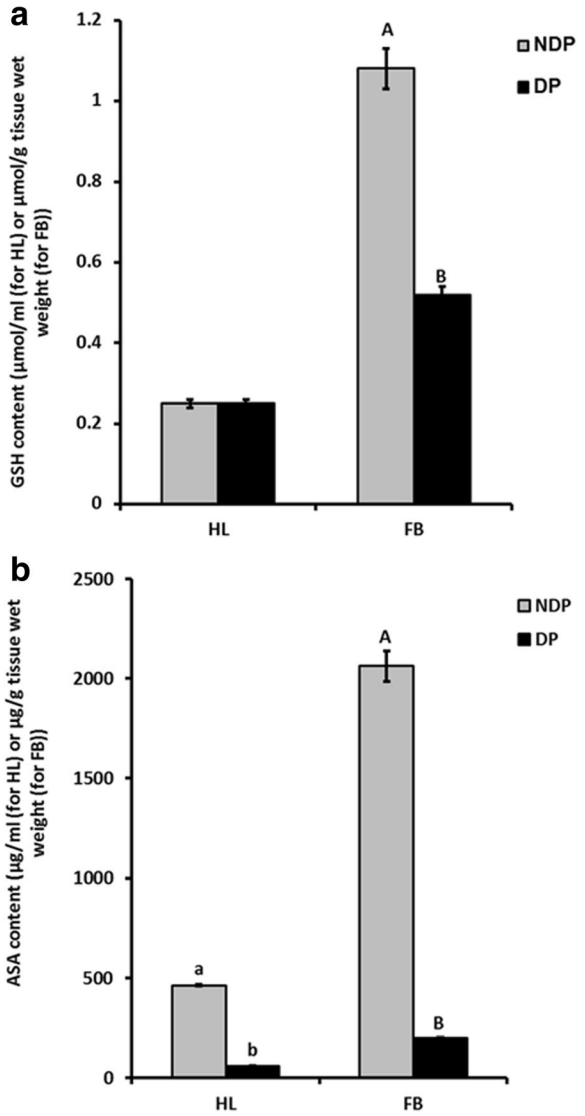


Fig. 3 a-b Non-enzymatic antioxidants. Content of GSH (**a**) in the hemolymph (HL) (µmol/ml) and fat body (FB) (µmol/g tissue wet wt.); and ascorbic acid (**b**) in the HL (µg/ml) and FB (µg/g tissue wet wt.) of pupae from nondiapausing (NDP) and diapausing (DP) generations. Bar represents mean ± S.E.M. ($n = 6$). Bars having different lower and upper case letters indicate significant difference in the measured parameters in HL and FB, respectively. Differences in the mean value have been considered statistically significant with a confidence interval of 95% and significant level of 5%

Conclusion

In summary, we observed that the fate of differential O_2 consumption and consequently the ROS metabolism are different in these two groups of pupae as evident from the low H_2O_2 pool in the diapause-destined pupa. In this

study we propose that low H_2O_2 content in diapause-destined pupa is associated with initiation of dormancy as reported earlier [8, 28, 30], along with higher activities of frontline antioxidant enzymes like SOD and CAT for enhanced oxidative resistance during extended lifespan [44, 46]. In contrast, higher GST activity and GSH content in the NDP group can be linked to rapid pupal development and an early metamorphosis (Fig. 4). Besides, higher levels of GSH and ASA content in NDP pupal tissues indicate a compensatory response to low SOD and CAT activities for the maintenance of redox status. Several mechanisms like changes in glutathione redox cycle [48], low H_2O_2 content [30], activation of extracellular regulated kinase (ERK) [55] and enhanced antioxidant protection [46] have been proposed as determining factors for dormancy and for an extended lifespan of diapause. However, ROS detoxification mechanism is well-known for the maintenance of homeostasis under such conditions.

The present findings, based on a naturally occurring phenomenon, in this silkworm species along with the previous reports brace up the conceptual framework for interdependence of ROS, quiescence, oxidative stress tolerance and extended lifespan. We envisage that future investigations should be carried out with an integrated approach for a comprehensive understanding of the above interactions and a possible crosstalk of the aforesaid components during insect development and diapause.

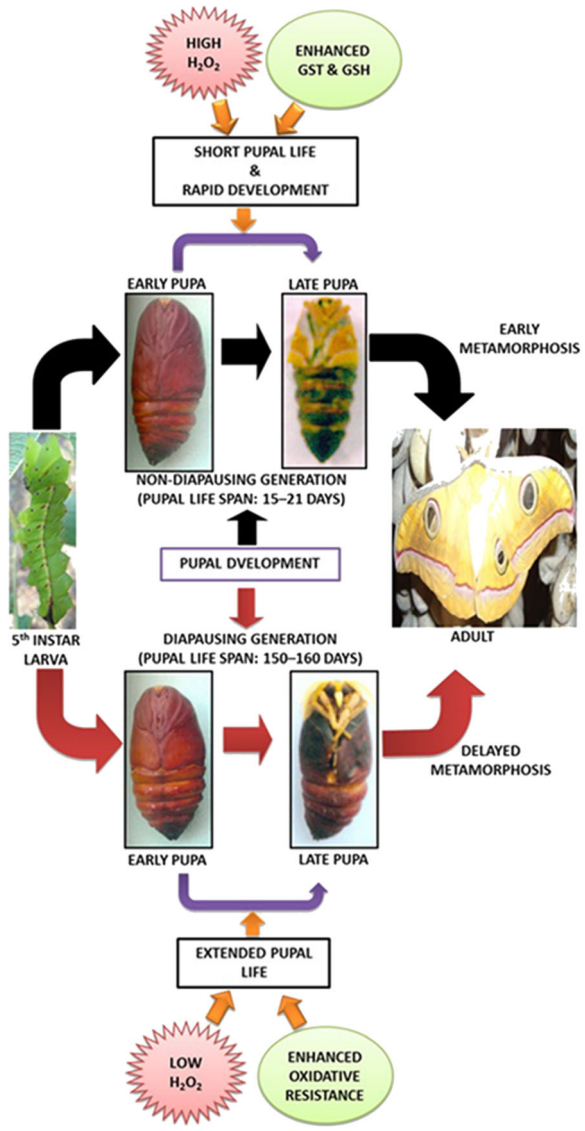


Fig. 4 Proposed mechanism of reactive oxygen species-mediated oxidative resistance and life span extension during pupal development of *Antheraea mylitta*

Additional file

Additional file 1: Representative excel data sheet for the respective figures. Data for each figure in the excel sheet provides the individual value, mean value, standard deviation (STDEV) and standard error of mean (SEM) for the measured parameters. Each figure is drawn based on the mean data and standard error of mean. (XLS 38 kb)

Abbreviations

ASA: Ascorbic acid; CAT: Catalase; CDNB: 1-Chloro-2,4-dinitrobenzene; DNPH: 2,4-dinitrophenyl hydrazine; DP: Diapausing; DTNB: 5,5′-Dithiobis-2-nitrobenzoic acid; EDTA: Ethylenediaminetetraacetic acid; FB: Fat body; GSH: Reduced glutathione; GST: Glutathione S-transferase; H_2O_2: Hydrogen peroxide; HL: Hemolymph; LPx: Lipid peroxidation; MDA: Malondialdehyde; NDP: Nondiapausing; PC: Protein carbonyl; PTU: Phenylthiourea; ROS: Reactive oxygen species; SOD: Superoxide dismutase

Acknowledgements

Authors 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 which enabled us to complete this work. Financial assistance from Department of Biotechnology, Govt. of India to the P.G. Department of Biotechnology, Utkal University is gratefully acknowledged for the development of basic infrastructure. We are sincerely grateful to Dr. Jatin K. Nayak, Retired Professor, Department of English, Utkal University, Bhubaneswar, for language correction and copy editing this manuscript.

Funding

Results embodied in this manuscript are not a part of any funded research project.

Availability of data and materials

All data generated or analysed during this study are included in this published article and available on line as supplementary material (Additional file 1).

Authors' contributions

JD and LS have designed the experiment. ALS has conducted the experiment. AD has analysed the data and developed the manuscript. All authors read and approved the final manuscript.

Ethics approval

All experiments have been conducted as per the guidelines of the Institutional Animal Ethics Committee, Department of Zoology, Utkal University, Bhubaneswar, Odisha, India. However, the insect species used in this study is reared for commercial production of raw silk materials, as a part of agro-based industry. Therefore, use of this animal in research does not require ethical clearance. We have obtained permission from the office of

Research officer sericulture, Baripada, Orissa, India for the provision of infrastructure and support for rearing of silkworm both in indoor and outdoor conditions related to our study to promote sericulture practices.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

Publisher's Note

Author details
[1]Department of Zoology, North Orissa University, Baripada, Odisha 757001, India. [2]Department of Biotechnology, Utkal University, Bhubaneswar, Odisha 751004, India. [3]Department of Zoology, Ravenshaw University, Cuttack, Odisha 753003, India.

Received: 21 August 2017 Accepted: 11 January 2018
Published online: 22 January 2018

References

1. Storey KB. Life in limbo: insights into the biochemical regulation of dormancy. Biochem Cell Biol. 1990;68:401–3.
2. Denlinger DL. Regulation of diapause. Annu Rev Entomol. 2002;47:93–122.
3. Denlinger DL. Why study diapause? Entomol Res. 2008;38:1–9.
4. Koštál V. Eco-physiological phases of insect diapause. J Insect Physiol. 2006; 52:113–27.
5. Mishra PK, Kumar D, Jaiswal L, Kumar A, Singh BMK, Sharan SK, Pandey JP, Prasad BC. Role of hemolymph biochemical constituents and brain proteins in maintenance of pupal diapause in Daba bivoltine ecorace of tropical tasar silkworm *Antheraea mylitta* Drury. J Ecophysiol Occup Hlth. 2009;9:243–52.
6. Jovanović-Galović A, Blagojević DP, Grubor-Lajšić G, Worland R, Spasić MB. Role of antioxidant defense during different stages of preadult life cycle in European corn borer (*Ostrinia nubilalis*, Hubn.): diapause and metamorphosis. Arch Insect Biochem Physiol. 2004;55:79–89.
7. Sohal RS, Agarwal S, Dubey A, Orr WC. Protein oxidative damage is associated with life expectancy of houseflies. Proc Natl Acad Sci. 1993; 90:7255–9.
8. Felton GW, Summers CB. Antioxidant systems in insects. Arch Insect Biochem Physiol. 1995;29:187–97.
9. Zhao LC, Sima YH, Shen XM. Metabolism of hydrogen peroxide in the course of embryonic development in silkworm. Dev Reprod Biol. 1999;8:41–8.
10. Zhao L, Shi L. Metabolism of hydrogen peroxide in univoltine and polyvoltine strains of silkworm (*Bombyx mori*). Comp Biochem Physiol B Biochem Mol Biol. 2009;152:339–45.
11. Hernández-García D, Wood CD, Castro-Obregón S, Covarrubias L. Reactive oxygen species: a radical role in development? Free Radic Biol Med. 2010; 49:130–43.
12. Allen RG, Sohal RS. Role of glutathione in the aging and development of insects. In: Collatz KG, Sohal RS, editors. Insect aging. Berlin: Springer Berlin Heidelberg; 1986. p. 168–81.
13. Allen RG, Venkatraj VS. Oxidants and antioxidants in development and differentiation. J Nutr. 1992;122:631–5.
14. Dmochowska-Ślęzak K, Fliszkiewicz M, Giejdasz K, Żółtowska K. The antioxidant system in diapausing and active red mason bee *Osmia bicornis*. Physiol Entomol. 2015;40:82–9.
15. Satapathy S, Mittra A. A comparative study on the metabolic rates in the pupae of the three voltine varieties of the tropical tasar silkworm *Antheraea mylitta* Drury. Sericologia. 2001;41:47–56.
16. Rath SS, Negi BBS, Singh BMK, Thangavelu K. Rate of oxygen uptake in *Antheraea mylitta* in various stages of development and during diapause. Int J Indust Entomol. 2005;10:45–9.
17. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 3rd ed. New York: Oxford University press; 2001.
18. Pick E, Keisari Y. Superoxide anion and hydrogen peroxide production by chemically elicited peritoneal macrophages—induction by multiple nonphagocytic stimuli. Cell Immunol. 1981;59:301–18.
19. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979;95:351–8.
20. Wills ED. Lipid peroxide formation in microsomes. General considerations. Biochem J. 1969;113:315–24.
21. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 1990;186:464–78.
22. Das K, Samanta L, Chainy GBN. A modified spectrophotometric assay of superoxide dismutase using nitrite formation by superoxide radicals. Indian J Biochem Biophys. 2000;37:201–4.
23. Sahoo A, Sahu S, Dandapat J, Samanta L. Pro-oxidative challenges and antioxidant protection during larval development of non-mulberry silkworm, *Antheraea mylitta* (Lepidoptera: Saturniidae). Italian J Zool. 2016;83:3–14.
24. Aebi H. Catalase. In: Bergmeyer HU, editor. Methods in enzymatic analysis, Vol. II. Academic Press: New York; 1974. p. 673–8.
25. Habig WH, Jakoby WB. Glutathione S-transferases (rat and human). Methods Enzymol. 1981;77:218–31.
26. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959;82:70–7.
27. Jagota SK, Dani HM. A new colorimetric technique for the estimation of vitamin C using Folin phenol reagent. Anal Biochem. 1982;127:178–82.
28. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193:265–75.
29. Zhao LC, Li B, Sima YH, Shen XM. Effects of hydrochloric acid on the metabolism of hydrogen peroxide during embryonic development of the silkworm, *Bombyx mori*. Acta Sericol Sin. 2000;26:268–70.
30. Jena K, Kar PK, Babu CS, Giri S, Singh SS, Prasad SS. Comparative study of total hydroperoxides and antioxidant defense system in the Indian tropical tasar silkworm, *Antheraea mylitta*, in diapausing and non-diapausing generations. J Insect Sci. 2013;13:123. https://doi.org/10.1673/031.013.12301.
31. Shen AY, Liu HT, Zhao LC. Activation of diapause eggs of the silkworm, *Bombyx mori* by exogenous hydrogen peroxide. Acta Sericol Sin. 2003;29:311–3.
32. Robbins HM, Van Stappen G, Sorgeloos P, Sung YY, MacRae TH, Bossier P. Diapause termination and development of encysted Artemia embryos: roles for nitric oxide and hydrogen peroxide. J Exp Biol. 2010;213:1464–70.
33. Barja G, Herrero A. Oxidative damage to mitochondrial DNA is inversely related to maximum life span in the heart and brain of mammals. FASEB J. 2000;14:312–8.
34. Barros MH, Bandy B, Tahara EB, Kowaltowski AJ. Higher respiratory activity decreases mitochondrial reactive oxygen release and increases life span in *Saccharomyces cerevisiae*. J Biol Chem. 2004;279:49883–8.
35. Barja G. Mitochondrial oxygen consumption and reactive oxygen species production are independently modulated: implications for aging studies. Rejuvenation Res. 2007;10:215–24.
36. Salin K, Auer SK, Rudolf AM, Anderson GJ, Cairns AG, Mullen W, Hartley RC, Selman C, Metcalfe NB. Individuals with higher metabolic rates have lower levels of reactive oxygen species in vivo. Biol Lett. 2015;11:20150538.
37. Aruoma OI. Free radicals, oxidative stress, and antioxidants in human health and disease. J Amer Oil Chem Soc. 1998;75:199–212.
38. Holmblad T, Söderhäll K. Cell adhesion molecules and antioxidative enzymes in a crustacean, possible role in immunity. Aquaculture. 1999; 172:111–23.
39. Patra B, Sahu S, Dandapat J. Effect of aminotriazole on antioxidant defense system of tasar silkworm *Antheraea mylitta*. Entomol Sci. 2014;17:316–23.
40. Khani A, Moharramipour S, Barzegar M, NADERI-MANESH HOSSEIN. Comparison of fatty acid composition in total lipid of diapause and non-diapause larvae of *Cydia pomonella* (Lepidoptera: Tortricidae). Insect Sci. 2007;14:125–31.
41. Saygili EI, Konukoglu D, Papila C, Akcay T. Levels of plasma vitamin E, vitamin C, TBARS, and cholesterol in male patients with colorectal tumors. Biochem Mosc. 2003;68:325–8.
42. Stadtman ER. Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. Free Radic Biol Med. 1990;9:315–25.
43. Ristow M, Schmeisser S. Extending life span by increasing oxidative stress. Free Radic Biol Med. 2011;51:327–36.
44. Orr WC, Sohal RS. Extension of life-span by overexpression of superoxide dismutase and catalase in *Drosophila melanogaster*. Science. 1994;263:1128–30.
45. Sim C, Denlinger DL. Catalase and superoxide dismutase-2 enhance survival and protect ovaries during overwintering diapause in the mosquito *Culex pipiens*. J Insect Physiol. 2011;57:628–34.

46. Tasaki E, Kobayashi K, Matsuura K, Luchi Y. An efficient antioxidant system in a long-lived termite queen. PLoS One. 2017;12:e0167412. https://doi.org/10.1371/journal.pone.0167412.
47. Meng G, Yao J, Wang L, Zhao L. Variation in glutathione status associated with induction and initiation of diapause in eggs of the bivoltine strain of the silkworm *Bombyx mori*. Physiol Entomol. 2011;36:173–9.
48. Zhao LC, Hou YS, Sima YH. Changes in glutathione redox cycle during diapause determination and termination in the bivoltine silkworm, *Bombyx mori*. Insect Sci. 2014;21:39–46.
49. Simon JY. Insect glutathione S-transferases. Zool Studies. 1996;35:9–19.
50. Ahmad S, Duval DL, Weinhold LC, Pardini RS. Cabbage looper antioxidant enzymes: tissue specificity. Insect Biochem. 1991;21:563–72.
51. Konno Y, Shishido T. Distribution of Gluathione S-transferase activity in insect tissues. Appl Entomol Zool. 1992;27:391–7.
52. Krishnan N, Kodrík D. Antioxidant enzymes in *Spodoptera littoralis* (Boisduval): are they enhanced to protect gut tissues during oxidative stress? J Insect Physiol. 2006;52:11–20.
53. Blagojevic D. The role of antioxidant protection in senescence of the bean weevil. University of Belgrade: Ph.D. thesis; 1995.
54. Gardiner CS, Reed DJ. Synthesis of glutathione in the preimplantation mouse embryo. Arch Biochem Biophys. 1995;318:30–6.
55. Fujiwara Y, Shindome C, Takeda M, Shiomi K. The role of ERK and p38 MAPK signaling cascades on embryonic diapause initiation and termination of the silkworm, *Bombyx mori*. Insect Biochem Mol Biol. 2006;36:47–53.