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OXYGEN CONSUMPTION AND ANTI-OXIDANT RESPONSE IN DABA AND LARIA ECORACES OF TROPICAL TASAR SILKWORM *ANTHERAEA MYLITTA* DRURY

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ABSTRACT
Wild tropical silkworm species *Antheraea mylitta* Drury commonly known as tasar silkworm is extensively exploiting for commercial silk production in India. This sericigenous insect species expresses divergent phenotypic characters in response to varying ecological and climatic conditions thus exist as ecoraces. Accordingly, ecoraces have acquired diverse productive traits such as cocoon characters, silk quality and quantity. In the present study, two different populations of wild ecorace Laria (Lohardaga & Bero) and semi-domesticated Daba were selected during pupal stage and studied the differential consumption of oxygen, oxidants and antioxidants in the pupal samples. The results showed significant variations ($p<0.001$) in the oxygen consumption rate in pupa of different populations studied. Higher production of oxidants *i.e.*, H_2O_2 (0.461mmol/mg protein) and lipid peroxidation (0.598 nmol TBARS/mg protein) in the Laria population (Bero) compared other Laria (Lohardaga) population and Daba (control). Correspondingly higher antioxidative enzymes (*CAT* and *GST*) recorded in the Laria (Bero) population besides, significantly ($p<0.05$) higher ascorbic acid content. The correlation between oxygen intake and hydrogen peroxide was very high and significant in Laria (Bero) at $p<0.001$. On the other hand, the same was not found to be significant in Laria (Lohardaga) and Daba. The correlation between H_2O_2 and LPX was significant at 5% level for Laria (Lohardaga) and Daba, while it was significant at 1% level for Laria (Bero). The study infers that, both the Laria populations are not similar with respect to physiological state. The information generated can be utilized in selection of Laria cocoons which will help in minimizing unseasonal emergence during winter, so as to build up stock for seed crop grainage.

KEYWORDS: Laria, Daba, Catalase, *GST, LPX,* Oxygen consumption, *Antheraea mylitta*

INTRODUCTION

Tropical tasar silkworm *Antheraea mylitta* Drury is an important wild sericigenous insect species, polyphagous in nature and reared outdoor on various food plants. Food plants of tropical tasar have been categorized as primary, secondary and tertiary on the basis of preferential feeding and/or adaptability of these silkworms on them (Singh and Srivastava, 1997). About forty four ecoraces have been identified with significantly divergent phenotypic characters in response to varying ecological and climatic conditions, thus exist as 'ecoraces' (Suryanarayana and Srivastava., 2005). Three important food plants such as *Terminalia arjuna* (Arjun), *Terminalia tomentosa* (Asan) and *Shorea robusta* (Sal) are widely utilizing for the rearing and production of commercial tasar silk. The implication of food plants on growth and development of the silkworms and subsequent productivity in terms of seed (fecundity) and silk recovery are prominent. Also it is apparent that the expression of phenotypic characters largely depends on interaction with its host plants biochemical profile and the environment (Srivastava *et al*., 2002). This aspect is largely influencing the rearing performance of tasar silkworms on different food plants belonging to the genus *Terminalia, Shorea, Zizyphus* (Suryanarayana *et al*., 2005). It is reported that the tasar silkworm reared on *Shorea robusta* (Sal) are more prone to virosis, leads to a significant crop loss. Moreover, there is other biotic and abiotic stress factor that might have been operating on silkworm when fed on different food plant. Insect populations are subjected to varieties of stress such as abiotic and biotic, fasting or nutritional deficiencies, xenobiotic compounds and toxic plant secondary metabolites, which generates reactive oxygen species (ROS). The cells have many ways to alleviate the effects of oxidative stress, either by repairing the damage or by directly diminishing the occurrence of oxidative damage by means of enzymatic and non-enzymatic antioxidants. If not eliminated, ROS propagate further oxidative process, leading to damage of cellular molecules, and resulting in disturbed homeostasis and cellular death. On the other hand, in insects ROS plays a significant role in the immunity, cell growth and apoptosis pathway (Kumar *et al*., 2003; Hao *at al*., 2003; Suzuki *at al*., 1997). It is indeed of immense relevance to study antioxidant defence in silkworm in wild environments. Herbivorous insect species are constantly challenged with ROS, which generated through endogenous (during normal metabolic pathway) and exogenously, such as exposure to radiations, pesticides, and phytochemicals (Jovanovic-Galovic *at al*., 2004; Zhao and Shi, 2009). Metabolisms of poikilothermic organisms, as expressed by oxygen consumption vary directly with fluctuation in the environmental temperature. The respiratory rate has a linear relationship with body weight and it varies with

temperature, season, age and oxygen concentration (Keister and Buck, 1964). The oxygen consumption in diapausing insects remains high during phases of morphogenesis but low during the period of diapause development (Wigglesworth, 1982). Depression in metabolism is always striking characteristic of diapause in many insects (Keister and Buck, 1964; Stegwee, 1964; Wigglesworth, 1982).

Daba is the bivoltine ecorace of *A. mylitta* with almost clear diapause induction and termination phenomenon. On the other hand, Laria is a Sal based wild ecorace in which the diapause phenomenon is not clearly understood. This ecorace behaves as uni, bi, tri-voltine in nature. In some of the populations of Laria have recorded unseasonal emergence of silk moths (eclosion) and continues even during winter period. The emergence takes place even to the tune of 50 to 60%. It is known that metabolism remains at the lowest during the diapause period of tasar silkworm and in many other insects. So, it is pertinent to say that the oxygen consumption during this period also remains at the lowest. Therefore, in the present study, the rate of respiration (oxygen consumption) was compared in different populations of tasar silkworm and simultaneously assessed the hydrogen peroxide production and lipid peroxidation as it seems that they are linked with oxidative metabolism of living organisms.

MATERIALS & METHODS

Two populations of Laria ecorace have been selected, the cocoons were collected after Laria silkworms rearing was completed on Sal plants from two different locations (Bero and Lohardaga area of Jharkhand State, India). Similarly, the cocoons of Daba bivoltine ecorace of tasar silkworm were collected from field Germplasm bank of Central Tasar Research & Training Institute, Ranchi, India for the study during the month of January. The pupae were cut open from the cocoon shell and used for analysis. Pupal weight was recorded in an electronic balance.

Oxygen consumption assessment

A respirometer or Warburg's apparatus was designed for the purpose. Pupae were kept inside for 4 hours and the rate of oxygen intake was calculated as ml/g live tissue/h. The displacement of blue colour (bromophenol blue) solution in the measurable syringe was recorded which was equivalent to the oxygen intake. For absorption of CO_2, sufficient quantity of KOH solution soaked in blotting paper was kept inside the conical flask, where pupae were kept.

Sample preparation

After the oxygen consumption assessment, the same pupae were used for biochemical studies. Pupae were dissected out, fat body samples were collected and homogenized with 1ml of 0.1 M phosphate buffer (pH 7). The homogenates from both preparations were separately transferred to 1.5mL centrifuge tubes and centrifuged at 15000×g for 20 min at 4°C. The centrifugation was done twice and supernatants were stored at -20°C for subsequent analyses.

BIOCHEMICAL ANALYSIS

Lipid peroxidation assay

The level of lipid peroxidation (LPX) was measured in terms of malondialdehyde (MDA), a product of LPX estimated by thiobarbituric acid (TBA) reaction (Heath and Packer, 1968). Fat body sample (0.5 g) was homogenized in 10 ml of 0.1% (*W*/*V*) trichloroacetic acid (TCA), and the homogenate was centrifuged at 7000 x *g* for 10 min. The supernatant was mixed with 0.5% TBA solution. Then the mixture was heated at 95°C for 45 min and cooled under room temperature. The supernatant was read at 532nm after removal of any interfering substances by centrifuging at 4000x *g* for 10 min. The amount of thiobarbituric acid reactive substances (TBARS) formed was calculated by using an extinction coefficient of $1.56x10^5(mol/L)^{-1}$ cm^{-1} (Wills, 1969), and expressed as nmol TBARS/g weight (wt) tissue.

Estimation of total hydroperoxides in silkworm fat body

Total hydroperoxides were determined spectro-photometrically according to the method of ferrous oxidation with xylenol orange (FOX1) (Wolff, 1994). 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 4000xg for 10min. The signal was read against an H_2O_2 standard curve and unit was expressed as µM / mg protein.

Catalase assay

CAT activity was determined according to Aebi (1974). The method is based on the decomposition rate of H_2O_2 by the enzyme. The assay mixture contained 20 mM H_2O_2 and sample (100µg protein). Absorbance was measured at 240 nm and CAT activity was expressed as nkat/ mg protein (1 katal = 1 mol sec^{-1}).

Glutathione-S-transferase assay

Glutathione-S-transferase activity was measured according to Habig *et al*. (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. Assay mixture contained 100 mM phosphate buffer (pH 7.0), 30 mM GSH, 15 mM CDNB and sample (100µg 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}.

Assay for Ascorbic acid

Samples were precipitated in 5% (w/ v) TCA in an ice bath and centrifuged at 5000 x g for 10 min. The deproteinised supernatant was used for the estimation of ASA. Ascorbic acid content was determined according to the method of Mitusi and Ohata (1961). The reaction mixture contained 2 % sodium molybdate, 0.15 N H_2SO_4, 1.5 mM Na_2HPO_4 and sample. The mixture was boiled at 90 ^oC for 45 min and then centrifuged at 1,000 x g 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/ g tissues.

Statistical analysis

Results are expressed as mean ± standard error of mean (SEM). Significant variations in the parameters were investigated by using one-way ANOVA. Differences were considered statistically significant when $p < 0.05$. Tukey's post hoc test was used to establish the HSD or CD among

the mean values. Regression and correlation analyses were also performed for different parameters in three tasar population samples. All the analyses were carried out by using MS-Excel software package.

RESULTS

Pupal weight

Before analyzing the oxygen consumption, the pupal weight was taken and the data is presented in Table 1. The pupal weight of Laria from Lohardaga was similar to that from Bero. The mean values of male and female pupae of Lohardaga population were 6.637 ± 0.806g and 7.677 ± 0.717g respectively and the values for Bero populations were 6.877 ± 0.493g and 8.08 ± 0.751g respectively. But the Daba (BV) recorded higher pupal weight . The variation for pupal weight among populations was high and significant (F = 35.36, P<0.001, CD = 4.024).

TABLE 1. Mean pupal weight and oxygen intake in two Laria populations and Daba ecorace in diapausing generation

Parameter	Sex	Laria (Lohardaga)	Laria (Bero)	Daba (Bivoltine)	F value	CD at 5%
Pupal weight (g)	Male	6.637 ±0.806	6.877 ±0.493	10.723 ±0.602	35.360	4.024
	Female	7.677 ±0.717	8.080 ±0.751	12.210 ±0.576	57.759	4.822
Oxygen intake (ml/g/h)	Male	0.014 ±0.002	0.060 ±0.007	0.026 ±0.004	63.681	0.043
	Female	0.026 ±0.002	0.088 ±0.008	0.032 ±0.006	138.230	0.062

Oxygen consumption

Although the pupal weight of two Laria populations was identical, the oxygen intake in Laria (Bero) was unusually high with a rate of 0.06 ± 0.007 and 0.088 ± 0.008 ml/g body weight/h in male and female pupa respectively, which was almost four times higher than that of other two populations. Oxygen intake in Laria (Lohardaga) was at par with that of Daba (BV) with value of 0.014 ± 0.002 and 0.026 ± 0.002 ml/g body weight/h respectively for male and female pupae (Table 1) ANOVA revealed highly significant variation among the three populations for oxygen consumption rate (F=57.579, P < 0.001, CD = 4.822).

Total hydroperoxide content

Total hydroperoxide content in both males and females the hydroperoxide content in Laria (Lohardaga) was at par with that of Daba (BV). The value in the males and females of Laria (Lohardaga) were 0.242 ± 0.038 and 0.288 ± 0.031 nmol/mg protein fresh tissue respectively. The same for Daba (BV) were 0.203 ± 0.035 and 0.325 ± 0.022 nmol/mg protein fresh tissue respectively. In Laria (Bero) hydroperoxide content was high with a value of 0.461 ± 0.032 and 0.473 ± 0.01 nmol/mg fresh tissue respectively in male and females. In case of all the populations, females recorded higher values in comparison to their male counterparts. Highly significant variation was observed among the three populations for hydroperoxide (Male: F = 15.724, P < 0.01, CD = 0.201; Female: F= 18.779, P < 0.01, CD = 0.141).

Lipid peroxidation

Lipid peroxidation (LPX) level in Laria (Lohardaga) was recorded to be 0.397 ± 0.028 and 0.297 ± 0.051 nmol TBARS/mg protein in males and females respectively. The values for Daba (BV) were 0.328 ± 0.054 and 0.330 ± 0.022 nmol TBARS/mg protein. Both these values were statistically comparable. On the other hand Laria (Bero) experienced a high level of lipid peroxidation and the values for males and females were 0.598 ± 0.049 and 0.991 ± 0.162 nmol TBARS/mg protein respectively (Table 2). The variation for lipid peroxidation among three populations was high and significant (Male: F = 9.736, P < 0.05, CD = 0.203; Female: F= 15.743, P < 0.01, CD = 0.567).

Catalase (CAT) activity

Among the anti-oxidative enzymes, catalase (CAT) activity showed significantly higher value for Laria (Bero) (Table 2). In case of males, the value was 273.704 ± 26.586 µkat/mg protein and in females 156.565 ± 15.7 µkat/mg protein. However, in both Laria (Lohardaga) and Daba (BV) the values were comparable for males as well as females. The variation for catalase activity among three populations was high and significant (Male: F = 8.111, P < 0.05, CD = 97.26; Female: F= 4.475, P < 0.05, CD = 43.566).

Glutathione-S-Transferase (GST) activity

Lowest activity of Glutathione-S- transferase (GST) was recorded in the Laria (Lohardaga) pupae (Table 2). Increased activity was recorded in Daba (BV) males and still higher significant increase in Laria (Bero). The F value was significant only in case of females (F = 0.431, P < 0.01 and CD = 16.574).

Ascorbic Acid content (ASA) content

Ascorbic acid (ASA) content level also showed the similar trend as shown by GST (Table 2). The variance was significant (P < 0.05) with F value of more than 5.00 and significant CD values.

TABLE 2. Oxidant and anti-oxidant status in two Laria populations and Daba ecorace in diapausing generation

Parameter	Sex	Laria (Lohardaga)	Laria (Bero)	Daba (Bivoltine)	F value	CD at 5%
Hydroperoxides (nmol/mg protein)	Male	0.242 ±0.038	0.461 ±0.032	0.203 ±0.035	15.724**	0.201
	Female	0.288 ±0.031	0.473 ±0.010	0.325 ±0.022	18.779**	0.141
Lipid peroxidation (nmol TBARS/mg protein)	Male	0.397 ±0.028	0.598 ±0.049	0.328 ±0.054	9.736*	0.203
	Female	0.297 ±0.051	0.991 ±0.162	0.330 ±0.022	15.743**	0.567
Catalase activity (µkat/mg protein)	Male	161.490 ±25.448	273.704 ±26.586	153.654 ±17.719	8.111*	97.260
	Female	115.503 ±16.198	156.565 ±15.700	97.949 ±9.887	4.475*	43.566
Glutathione-S-transferase (nmol CDNB formed/ min/mg protein)	Male	89.106 ±5.927	110.797 ±3.541	103.904 ±12.013	1.919	6.051
	Female	38.361 ±5.706	54.744 ±3.741	32.755 ±3.783	6.431**	16.574
Ascorbic acid (µg/mg protein)	Male	3.834 ±0.325	5.592 ±0.695	3.489 ±0.384	5.182*	1.633
	Female	1.558 ±0.243	2.287 ±0.162	1.406 ±0.189	5.487*	0.682

The relationship among different parameters was also studied through linear regression and correlation analysis. The correlation coefficient (r) values for pupal weight with oxygen intake, lipid peroxidation and hydrogen peroxide content was highly significant in all the three populations studied (Table 3). The correlation between oxygen intake and hydrogen peroxide was very high and significant in Laria (Bero) ($P < 0.01$). On the other hand, the same was not found to be significant in Laria (Lohardaga) and Daba (BV). The correlation between H_2O_2 and LPX was significant at 5% level for Laria (Lohardaga) and Daba (BV) while it was significant at 1% level for Laria (Bero).

TABLE 3. Correlation coefficients among different traits studied

Traits	Laria (Lohardaga)	Laria (Bero)	Daba (Bivoltine)
Pupal weight vs. Oxygen intake	0.892**	0.879**	0.842**
Pupal weight vs. Lipid peroxidation	0.903**	0.965**	0.867**
Pupal weight vs. Hydroperoxide	0.854**	0.929**	0.797*
Oxygen intake vs. Hydroperoxide	0.601	0.923**	0.660
Lipid peroxidation vs Hydroperoxide	0.855*	0.929**	0.767*

NB. ** Significant at 1% level

DISCUSSION

Insects, being poikilothermic organisms, their metabolism as expressed by oxygen consumption vary directly with fluctuation in the environmental temperature. The respiratory rate has a linear relationship with body weight and it varies with temperature, season, age and oxygen concentration (Keister and Buck, 1964). Respiratory rate is greater in adult, than in larva while pupal consumption is lower than either (Chapman, 1998). Oxygen consumption in aerobic cells is accompanied by generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), lipid peroxides, and hydroxyl radicals. If not eliminated, ROS propagate further oxidative processes, leading to damage of cellular molecules, and resulting in disturbed homeostasis and cellular death. Furthermore, herbivorous insects are continuously exposed to ingested pro-oxidants (Pritsos *et al*., 1988), and the resulting oxidative injury could be attributed to H_2O_2–mediated effects (Peric´-Mataruga *et al*., 1997). Hydrogen peroxide–mediated lipid peroxidation in insect cells could have particularly devastating consequences and could thus influence the developmental process, since lipids have many important physiological functions. In the present study it was found that there was no difference in the pupal weight of two Laria populations. On the other hand there was marked difference in oxygen consumption, hydroperoxide and LPX content in the Bero population of Laria in comparison to Laria (Lohardaga) and Daba. This is an indicative of active state of metabolism in this population. In other words this also provides a clue for the hypothesis of improper diapause in Laria (Bero). The results indicate that the Laria population from Lohardaga behaves as Daba (BV) with identical parameters of oxygen intake as well as oxidant production in the form of hydroperoxide and LPX.

In general, oxygen consumption by diapausing insects is lower than that of corresponding active stages. There are

however, considerable differences between species. Mostly dormant stages of insects consume between 5 to 70% oxygen as the corresponding active stages do (Keister and Buck, 1964; Hayes *et al*., 1968; Adamek and Fisher, 1985). Thus, the reduction of oxygen consumption is sometimes used as evidence for the induction of a diapause (Modder, 1978; Gehrken, 1985). The finding of the present study also corroborates the above reports. The enhanced level of thiobarbituric acid reactive substances (TBARS) and total hydrogenperoxide levels in the Laria (Bedo) pupae were observed probably in response to emergence linked metabolism. Lipid peroxidation products are not only a marker of oxidative damage to lipids; but also involved in triggering the up regulation of antioxidant defense mechanisms (Lushchak and Bagnyukova, 2006c). Catalase (CAT) is principal H_2O_2 scavenging enzymes. In the present study, increase in activities of CAT was observed in Laria (Bero) pupa. The results indicated that the active state of metabolism in association with increased respiration rate might have resulted in elevation of hydroperoxide level in tissues, as a consequence of which CAT activities increased in concert to remove H_2O_2. The results are consistent with findings of An and Choi (2010) who have also observed increased CAT activity in gills and digestive glands of ark shell (*Scapharca broughtonii*) exposed to temperature. GST catalyzes the conjugation of reduced GSH to nucleophilic xenobiotics or cellular components damaged by ROS (Van der Oost *et al*., 2003). The increased activity of the enzyme during thermal stress in the present experiment suggests that GST is actively involved in removal of toxic LPX products accumulated. ASA is a strong antioxidant (Halliwell and Gutterdige, 2001), which contributes substantially to the ability of cells or tissues to cope with oxidative stress. In the present study a significant increase in ASA content was observed in Laria (Bero) pupa which may be due to the increased availability of GSH which reduces dehydroascorbate to ascorbate (Winkler *et al*., 1994). From the present study it is clear that both the Laria populations analyzed are not similar so far as their physiological state is concerned. However, more pursuasion is required especially on the expression pattern of metabolic enzymes as well as the enzymes involved in the anti-oxidation process. The Oxygen consumption, H_2O_2 production and lipid peroxidation can be used as an indicator for selection of Laria populations which will have fewer emergences during winter, so as to reduce the loss due to unseasonal emergence and to build up stock for next seed crop grainage.

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