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Higher concentrations of heavy metals impair antioxidant defense mechanism and growth response of muga silkworm, *Antheraea assamensis* (Lepidoptera: Saturniidae)

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Abstract
Oxidative stress leads to imbalance between the production of free radicals and antioxidant defense mechanism. Muga silkworm being reared outdoor is subjected to heavy metal pollution. The present study focuses on the potential capacity of larvae in terms of growth and antioxidant enzyme activity fed with heavy metals (Cd, Pb, Mn & Zn) stressed leaves *in vitro* condition. Heavy metal accumulation in pupa was correlated with body weight and instar duration and its effect on silk thread was studied. The body weight and instar duration was significantly varied in high dose of Cd and Pb, indicating that metal pollution prevented silkworm from achieving better growth. Effect of heavy metal on the antioxidant enzyme activity (Glutathione-S-transferase, superoxide dismutase, catalase) showed significant difference than control. Groups were compared by One-way ANOVA using Student Neumann Keul test. Present study suggests that activity of antioxidant enzymes in the larvae varies with respect to time and concentration of heavy metal ions.

Keywords: *Antheraea assamensis*, antioxidant enzymes, heavy metals, oxidative stress

1. Introduction
The safe clearance of industrial effluents is becoming a serious concern worldwide. Increase in industrialization and inputs of chemicals in agro-ecosystem leads to the accumulation of toxic metals in the air, soil and water bodies. Anthropogenic activities such as metalliferous mining, smelting, application of chemical fertilizers and natural activities had resulted in the significant accumulations of variety of heavy metals in terrestrial ecosystems [27]. Being non-biodegradable in nature, metals persist in the nature for long time and causes deleterious effects to both human health and environment. Heavy metals has relatively high density and toxic at low quantity, e.g., arsenic (As), lead (Pb), mercury (Hg), cadmium (Cd), chromium (Cr), thalium (Tl). Some trace elements are also considered as zinc (Zn), copper (Cu), selenium (Se). They are present mostly in soil and aquatic ecosystems rather than atmosphere. The toxicity of heavy metals is dependent on dose, route of exposure, chemical species, age, gender, genetics and nutritional status of exposed individuals [35]. They are important constituents of several key enzymes and various oxidation reduction reactions. Their negative effects includes increase of reactive oxygen species (ROS) for eg. superoxide anions, hydrogen peroxide and hydroxyl radicals [24]. Among heavy metals pollutants, cadmium (Cd) and lead (Pb) are not able to participate in metabolic reactions in herbivores [40]. Cd is absorbed by host plants and accumulated in phytophagous insects through the food chain inducing irreversible damages, ultimately leading to cell death. It is a cumulative toxicant and is of concern of phytophagous insects as it concentration tends to increase with age [36]. Deleterious effects of Cd are associated with alterations in the redox status of the cell. Scientific studies revealed the crytogenic, mutagenic and carcinogenic effects of cadmium on organisms including invertebrates [45]. It interferes with the antioxidative defense system, thereby resulting in the formation of free radicals and consequently lipid peroxidation. Cd accumulates in the animal tissues and disturb their physiological functions due to its non-degradable nature. Toxicological profile of Pb comprises generation of oxidative stress, ionic mechanism and apoptosis [16]. Exposure to lead causes many side effects depending on the level and duration. Zn is an essential element and play a vital function in protein, lipid and carbohydrate metabolism, however at higher concentrations it can induce a broad range of physiological,

biochemical, and behavioural dysfunction in herbivores [38].

Insects have a distinct role in trophic webs as prey of birds, mammals and other arthropods. Therefore, they are considered as an important path of bioaccumulation and their toxicological evaluations are interesting. Arthropods are able to detoxify metals, either by bounding to specific proteins such as metallothionines, or by compartmentation process within membrane lipid vesicles [5]. In insects, pollution induced effects reported were reduction in pupal weight [22], increase in relative growth rate (RGR) [46] and larval mortality [32]. Metals such as Cd, Cu, Zn and Ni gets accumulated in herbivorous insects when they consume metal exposed leaves [26]. High concentration of Ni in *Spodoptera litura* retarded the RGR and lowered the immune response while low concentration increased the RGR and encapsulation grade [42]. A contaminated ecosystem exerts a strong selection of populations based upon their metal tolerance capability [43]. There are many mechanisms present which protects organisms living in metalliferous areas or under stress conditions. The evolved tolerance to metals is due to the presence of effective antioxidant defence. Antioxidant defences protects living system from metal induced oxidative stress [13]. Antioxidant defense mechanism present in insects consists of antioxidant enzymes and low molecular mass components having biological role in neutralizing and suppressing oxidative damage. Enzymes are superoxide dismutase (SOD), catalase (CAT), isoforms of glutathione-S-transferase (GST) with peroxidase activity, glutathione reductase (GR), ascorbate peroxidase (APOX) and non-enzymatic cellular antioxidants glutathione (GSH), ascorbic acid etc. [4]. GSTs are ubiquitous in nature and involved in detoxification of xenobiotic compounds in vertebrates and invertebrates [37]. It catalyses the nucleophilic addition of the tripeptide glutathione to substrates, that have functional electrophilic functional groups. Glutathione levels and activity of glutathione dependent enzymes in insects living in the polluted environment have been shown in maintaining redox homeostasis and the intensity of enzyme response is dependent on developmental stage and state of nutrition [31]. Superoxide dismutase (SOD) and catalase (CAT) are front line antioxidant enzymes which limit the balance of superoxide radicals and H_2O_2 respectively [8]. Both enzyme maintains the balance of superoxide radicals and H_2O_2. As a scavenger SOD catalyses the dismutation of superoxide ($O_2^{\cdot}$) to hydrogen peroxide (H_2O_2) and oxygen (O_2). When larvae feeds on the plants containing heavy metals like Cd, Cu and Zn, the transport of metal from the plant to the adult is regulated [28]. *Lymantria dispar* L larvae when fed with cadmium enriched diet, a change in the larval duration, relative growth rate and mass were observed [11].

The Indian golden silkmoth (*Antheraea assamensis*, Lepidoptera: Saturniidae), popularly known as Muga silkmoth, is a semidomesticated, golden color silk producing insect, endemic to Northeast India. It is multivoltine, polyphagous, holometabolous sericogenic insect. The lustrous golden yellow colour and strong nature of the thread obtained from the Muga silkworm cocoon, are the two unique features. The traditional outdoor rearing system of Muga silkworm has many obstacles that includes not only attack by birds, predators and diseases, but also agricultural chemicals and pollutants form oil fields [5]. Polluting agents such as aliphatic-aromatic hydrocarbons and heavy metals present in the soil and leaves of *Machilus bombycina* located near to oil field area of North-East India, hampers the silk cocoon production [18]. Muga silkworm life cycle parameters, survivability, silk productivity and constituents of individual silk fibres has adversely effected in the silkworm present near vicinity of brick kilns, cement factory and oil refineries. The aim of the present study was to examine the effect of heavy metal doses (Cd, Pb, Mn & Zn) on antioxidant enzyme activity (GST, SOD & CAT) and growth related traits (instar duration & body weight) of Muga silkworm *in vitro* condition. The accumulation of heavy metals on the pupa was correlated with body weight and instar duration and its consecutive effect on silk thread. This study aims to test the hypothesis that heavy metals would alter the constitutive levels of antioxidant defence.

2. Materials and Methods

2.1 Selection of heavy metals

Heavy metals were selected based upon the concentration present at polluted and non-polluted site. One farm named as State Silkworm Concentration Centre located in Morigaon district, Assam (26.1608˚N, 92.2138˚E) was selected as polluted site due to continuous exposure of fumes from brick industries, Paper Mill of Hindustan Corporation Ltd. and nearby tea gardens. Similarly, non-polluted site was selected at CSIR-North East Institute of Science and Technology, Assam (26˚44’10”N, 94˚9’30”E) campus. Air samples from both sites were collected through glass microfiber filters of size 1.6µm with the help of Respirable Dust Sampler (Envirotech Model APM 460 DXNL).

2.2 Insect rearing and estimation of fitness related traits

Disease free layings of Muga silkworm were collected from Central Muga Eri Research & Training Institute, Jorhat, Assam (26.7953ºE, 94.3194ºN). Eggs were disinfected in 0.1% sodium hypochlorite for 5mins and rinsed with distilled water and air dried. Leaves of the host plant were treated with heavy metals Cd, Pb, Mn and Zn and the chemical sources were cadmium chloride ($CdCl_2$), lead chloride ($PbCl_2$), manganese chloride ($MnCl_2$) and zinc chloride ($ZnCl_2$), where water was used as a solvent. The respective conc. were $CdCl_2$ (0.0016, 0.02, 0.04 & 0.06), $PbCl_2$ (0.05, 0.07, 0.08 & 0.09 ppm), $MnCl_2$ (0.02, 0.03, 0.04 & 0.05 ppm) and $ZnCl_2$ (0.02, 0.04, 0.06 & 0.08 ppm). Fifth instar larva (n=30) with similar body weight and length were divided into five groups including control and allowed to fed on Cd, Pb, Mn and Zn stressed leaves. Control group was exposed to leaves having distilled water. Each treatment was performed in triplicates. The experiment was performed for three consecutive days and larval weight of each group were measured after treatment. Similarly, instar duration were also recorded.

2.3 Estimation of heavy metals in the pupal sample of treated and control larvae

The amount of heavy metals present in the pupa sample were measured using Atomic Absorption Spectroscopy (AAS) (Perkin Elmer, A-Analyst -700) [9]. Briefly, 10 nos. of pupae from each treatment were weighed and digested at a temperature of 210°C for 60mins using the acid mixture (nitric & perchloric acid in the ratio 1:2). The solution was boiled until a clear solution was formed and allowed to cool at room temperature. A series of standards were prepared accordingly, and the absorbance levels were compared with the experimental samples. Heavy metal accumulation on the pupa was correlated with the body weight and instar duration.

2.4 Preparation of homogenates

Effect of exposure on antioxidant enzyme activity (GST, SOD & CAT) were assayed. Larva were anthesized using liquid nitrogen. Frozen larva were kept at a temperature of -20 ºC till the homogenate preparation. Larvae were homogenised on ice containing 0.1 M phosphate buffer (pH 7) using an sonifier for 3 x 15s. Homogenates were centrifuged at 10,000 rpm for 15 mins (4ºC). The supernatant were extracted and frozen at -20°C for enzymatic studies.

2.5 Activity of antioxidant enzymes

2.5.1 Glutathione-S-Transferase assay

The GST activity was assayed using CDNB as substrate by the method of Habig *et al* [19]. The activity was monitored by change in absorbance at 340nm due to thioether formation at 25ºC. The reaction mixture contains 33µl of 50mM 1-chloro-2, 4-dintrobenzene (CDNB) (Sigma Aldrich, St. Louis, MO), 100µl of 50mM reduced glutathione (GSH) (Sigma Aldrich, St. Louis, MO) and 1850µl of sodium phosphate buffer (100mM, pH 6.5). Finally, 10µl of enzyme stock was added to the mixture. The reaction was carried out in triplicate. The mixture was incubated for 2-3 mins at room temperature and then transferred to 96 well microtiter plate. Reaction mixture (2ml) without enzyme extract was taken as blank and the readings were taken using micro plate reader (Bio Tek, USA). The GST activity was measured using the equation:

$$\text{GST activity} = (\Delta A_{340})/\text{min} \times V(\text{ml}) / \varepsilon_{Mm} \times V_{enz}(\text{ml})$$

where, mM (mM^{-1} cm^{-1}) – extinction coefficient for CDNB conjugate at 340 nm, V- the reaction volume, V_{enz}- the volume of the enzyme sample.

2.5.2 Superoxide dismutase assay

The SOD activity was measured using the method described by Marklund [30]. Reaction mixture contains 2.8ml of Tris-EDTA (50mM Tris and 10mM EDTA, pH 8.2) buffer and 50µl of enzyme supernatant. The reaction was initiated after the addition of pyrogallol (15mM). The rate of auto oxidation was measured at 440nm using micro plate reader (BioTek, USA) and the readings were taken for 3 mins. One unit of SOD activity is defined as amount of protein per milligrams causing 50% inhibition of pyrogallol autoxidation. The total SOD activity was expressed as units per milligrams of protein. The results were expressed using the equation:

$$\% \text{ Inhibition} = [(A_0-A_1)/A_0] \times 100$$

Where, A_0 is the absorbance of the blank, A_1 is the absorbance of test sample.

2.5.3 Catalase assay

The CAT activity was measured using the method of Sinha *et al* [41]. About 20µL of sample was mixed with 200µL of 5mM H_2O_2, and a time scan was performed for 1 min at 240 nm at 25°C. The disappearance of the peroxide depending on the catalase activity was observed. One unit of CAT activity is defined as 1µmol conversion of H_2O_2 per min/ml sample. CAT activity was calculated using the formula:

$$(\Delta A_{240}\,\text{min}) \times (1.4\text{ ml}) / (39.4) \times (\text{sample-initial})\ \mu\text{mol/ml/min}$$

2.6 Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX) studies

The impact of heavy metals $PbCl_2$ (0.09 ppm), $CdCl_2$ (0.06 ppm), $MnCl_2$ (0.05 ppm) and $ZnCl_2$ (0.08ppm) on the silk thread quality was analysed. The sample were mounted on metal stubs with an adhesive band for SEM analysis. The samples were coated gold and the electron micrographs of the specimen were taken by Carl Zeiss Microscopy (ZEISS SIGMA). EDX profile of the respective doses were also taken.

3. Statistical analysis

The results were processed using statistical package Sigma Plot 11.0 software (Jandel Scientific, San Rafael, CA). The results were expressed as the mean ± standard error (SE) of three replicates. Difference in the average value of control and treated group were assessed by one-way ANOVA and Student Neumann-Keul (SNK) test. Relationship between heavy metal accumulated in the pupa was correlated with body weight and instar duration. Results were analysed using Pearson Product moment correlation coefficient (R). In all cases, $P< 0.05$, $P<0.01$ or less were considered significant. All the assays were performed in triplicates.

4. Results and Discussions

4.1 Effect of heavy metals on fitness related traits

As a source of micronutrients insects need variety of minerals and trace elements. Generally, low concentrations of pollutants have positive effects on insects but increase in concentration have harmful effects [46]. On exposure to heavy metals larval mass reduces thereby leading to neuroendocrine/hormonal disruption [23]. Heavy metal exposure on instar duration and body weight is depicted in Table 1 & 2. Heavy metal exposure resulted in significant prolonging of the instar duration. In lower dose of Pb and Cd duration was not significant, whereas higher doses have significant effect to control. In contrast, Mn & Zn doses does not show significant changes. Stress influence the synthesis, secretion of ecdysteroids in insects and prolong their developmental durations [12]. Zn play a vital role in lipid synthesis, proteins, and carbohydrates in reducing the duration of larval and pupal stages [3]. Zn is involved in various metabolic reactions in insects, such as creating active enzymatic centers, organizing protein domains and participating in DNA synthesis, and play an important role in the growth and development of insects [15]. Larva strongly attained lesser body weight after exposure to Cd and Pb than control depicting that better growth on control leaves. Intoxification of Cd is correlated with the changes in synthetic and secretory activity of protocerebral neurosecretory cells, which regulates the level of morphogenetic hormones in hemolymph (juvenile and ecdysone) [23]. Mn dose not showed any significant changes to control. The body weight after Zn exposure increases in a dose dependent manner. Zn increases the weight of larva and serigene gland in mulberry silkworm [1]. A negative correlation was observed between the Cd, Pb and Mn accumulations on pupa to the body weight while positive correlation was observed for Zn (Fig. 1). R values were -0.863, -0.87, -0.822 and 0.937 for Cd, Pb, Mn and Zn. Positive correlation was obtained between the metal accumulation on pupa to the instar duration. R values were 0.961, 0.965, 0.901 and 0.945 for Cd, Pb, Mn and Zn (Fig. 2).

4.2 Antioxidant enzyme activity of *A. assamensis* larvae

The antioxidant enzyme activity in the treated and control group showed different pattern of response according to the doses used.

4.2.1 Glutathione-s-transferase activity

Glutathione is a low molecular weight thiol compound present in the cells and considered as a crucial component of antioxidant defense system. The oxidation of GSH is catalyzed by GST by protecting cells from oxidative injury. GST signifies the first line of defense in insects against free radicals and reflect the adaptation of insects to exposure with higher doses of toxic chemicals. Results clearly indicates that with increase in doses of heavy metals GST activity either increase or decrease depending upon the toxicity (Fig. 3). GST activity in the larva that fed on $CdCl_2$ stressed leaves was significantly lower in higher doses, whereas lower dose (0.0016ppm) has no effect on GST activity (Fig. 3A). GST activity reduces from 1st to 3rd day in all treatment groups. This suggests that apparent production of ROS inactivated the GST enzyme. Cummulative effect of Cd can inhibit the activity of glutathione-dependent enzymes including GST, through the inhibition of ɣ-glutamilcysteine synthetase and glutathione biosynthesis [7]. When Cd accumulates in the insect, it combines with the active center (Se-Cys) of GPx, leading to structural changes which inactivates GPx [48]. Higher concentration of cadmium in food destroy the midgut structure and impact fundamental processes (digestion and absorption of food) causing starvation effects and reduction in larval growth and survival [33, 47]. GST activity of the larva that fed on $PbCl_2$ stressed leaves was significantly increase in all treatment groups from 1st to 3rd day than that of control (Fig. 3B). GST activity after $MnCl_2$ treatment does not vary significantly during 3rd day of exposure in all treatment groups. However, with a dose of 0.04 ppm $MnCl_2$ activity found to be significantly different from the control group (Fig. 3C). GST activity was found to decreasing after $ZnCl_2$ treatment as the dose increases (Fig. 3D). However, a marked increase in activity was observed with dose 0.02 ppm during 2nd day from the control. Increase in GST activity at 2nd day after zinc treatment is correlated with the findings of Saliu and Bawa-Allah [39] and Farombi *et al.* [14]. They have reported that increased activity of GST and GSH amount in Zn treated African cat fish, *Clarias gariepinus.* Significant difference was observed among the larva treated with 0.04ppm from 1st to 3rd day.

4.2.2 Superoxide dismutase activity

SOD activity after the treatment of heavy metals was assayed from 1st to 3rd day of exposure (Fig. 4). As shown in Fig. 4(A) SOD activity of larva that fed on the $CdCl_2$ stressed leaves were significantly higher as the dose increases from 1st to 3rd day than that of control. The enhanced SOD activity might be in response to the superoxide radicals induced by the Cd stress and then converted harmful superoxide radicals into hydrogen peroxide via Haber-Weiss reaction [43]. SOD activity in the $PbCl_2$ treated group increases during 3rd day in each treatment than control. Also, significant difference ($p<0.01$) was observed among each treatment group from 1st to 3rd day (Fig. 4B). Activities of antioxidant enzymes may change radically during the start of an exposure to heavy metals, but then return to normal levels within a few days [20]. Exposure of larva to $MnCl_2$ treated leaves does not showed any significant difference in SOD activity in lower doses from 1st to 3rd day. However, SOD activity significantly differ at higher dose (0.09ppm) than control from 1st to 3rd day (Fig. 4C) suggesting that higher level of Mn-SOD diminishes the oxygen free radicals. Mn-SOD provides the cell's primary defenses against free radicals and it is necessary for maintaining a balance between oxidants and antioxidants [10]. SOD activity observed after $ZnCl_2$ treatment significantly increased than control. Activity in larva treated with 0.02ppm $ZnCl_2$ were not significantly different than that of untreated control (Fig. 4D). Zn acts an cofactor of enzymes SOD1 and SOD3 and it protects sulfhydryl groups from oxidative damage by binding directly to them and reduces reactivity of sulfhydryl group and thereby prevents the formation of intramolecular disulfide bond. Zn inhibits the ROS production by competing with transition metals, especially with Fe [6].

4.2.3 Catalase activity

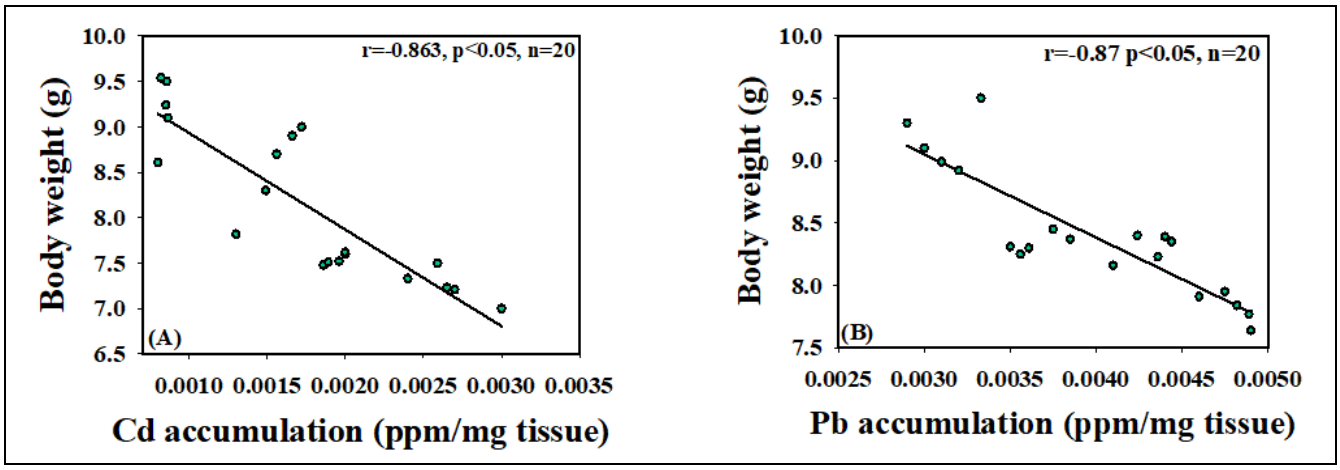
SOD and CAT are most important antioxidant enzymes that are functionally interconnected because SOD converts the superoxide free radical ($\cdot O^{2-}$) to H_2O_2, which is eliminated by CAT [29]. Results of this study clearly shows that SOD and CAT exhibit a coupled behaviour after $CdCl_2$ treatment, which is because of their physiological independence. Increase in activity of CAT and SOD is observed in the condition of environmental pollutants since SOD-CAT represents the first line of defense against oxidative stress [34]. CAT activity of the larva after exposure from 1st to 3rd day is presented in Fig. 5. CAT activity was found to be increase after $CdCl_2$ exposure from 1st to 3rd day (Fig. 5A). Increase in CAT activity is due to presence of peroxides and other damaging species. CAT activity is mainly induced by the presence of certain concentrations of peroxide in the body [21]. CAT activity was found to be decreased in the larva fed on $PbCl_2$ stressed leaves from 1st to 3rd day. CAT activity after $MnCl_2$ was significantly decreased from 1st to 3rd day as dose of treatments increases (Fig. 5C). There was no clear relation was obtained between the SOD and CAT activity in Mn treated groups. CAT activity after $ZnCl_2$ treatment showed no significant difference in lower doses than control group, indicating that accumulation of Zn did not increase ROS production. However, activity was significantly higher in the larva after the exposure of doses 0.06 & 0.08 ppm during 3rd day (Fig. 5D). Earlier findings demonstrates that concentrations of nutritional metals (eg. Zn) in the arthropods can be regulated more efficiently than those of non-essential metals [25]. Non-significant effect of Zn stress on antioxidant enzymes was might be due to non-enzymatic cellular antioxidants which can act as a substitute for antioxidant enzymes [17]. However, with an increase in concentration of $ZnCl_2$, CAT activity was found to significantly different in the 3rd day, suggesting that excessive Zn concentration can inhibit the antioxidant enzyme activity as did by nonessential metals. CAT activities were inhibited in the whole body tissue of the tasar silkworm larvae when exposed to Zn [38]. SEM analysis reveals that higher doses of $CdCl_2$ (0.06 ppm), $PbCl_2$ (0.09ppm), $MnCl_2$ (0.05ppm), $ZnCl_2$ (0.08ppm) accumulates on the silk thread thereby deteriorating the silk quality (Fig. 6A-E). EDX analysis showed the presence of respective heavy metal along with carbon and oxygen (Fig. 6a1-e1).

Table 1: Changes in body weight (g) of *A. assamensis* larva exposure to heavy metals. Symbols after the value represents significant difference ($p<0.01$) among the control group and various doses of treatments in the column.

Exposure conc. of heavy metals (ppm)	Body wet weight (g/larva)		
	Exposure time (day)		
	1st day	2nd day	3rd day
$PbCl_2$ group			
0.05	8.25 ± 0.35	8.65 ± 0.24*	8.92 ± 0.37
0.07	8.16 ± 0.15	8.24 ± 0.26*	8.31 ± 0.32*
0.08	8.04 ± 0.42	8.12 ± 0.16*	8.16 ± 0.28*
0.09	7.88 ± 0.41*	7.98 ± 0.23*	8.02 ± 0.24*
$CdCl_2$ group			
0.0016	8.15 ± 0.34	8.25 ± 0.35	8.61 ± 0.35*
0.02	7.56 ± 0.52*	7.77 ± 0.25*	7.82 ± 0.25
0.04	7.33 ± 0.41*	7.45 ± 0.41*	7.48 ± 0.21*
0.06	7.21 ± 0.49*	7.26 ± 0.26*	7.33 ± 0.19*
$MnCl_2$ group			
0.02	9.65 ± 0.43	9.88 ± 0.28	10.24 ± 0.43
0.03	9.54 ± 0.41	9.84± 0.41	10.16 ± 0.41
0.05	9.41 ± 0.52	9.79± 0.35	9.88 ± 0.32
0.04	9.32 ± 0.28	9.24 ± 0.25	9.45 ± 0.26
$ZnCl_2$ group			
0.02	9.65 ± 0.35	9.99 ± 0.25	10.39 ± 0.39
0.04	9.78 ± 0.65	10.36 ± 0.39	10.45 ± 0.41
0.06	9.84 ± 0.34	10.39 ± 0.41	10.49 ± 0.45
0.08	9.89 ± 0.24	10.47 ± 0.45	10.56 ± 0.52
Control	9.99 ± 0.25	10.35 ± 0.32	10.41 ± 0.45

Table 2: Changes in body weight (g) of *A. assamensis* larva exposure to heavy metals. Small letters after the value represents significant difference ($p<0.01$) among the control group and various doses of treatments in the column.

Treatments	Concentration of heavy metals (ppm)	Instar duration (days)
Control		5.66 ± 0.33
$PbCl_2$ group	0.05	7.33 ± 0.57
	0.07	7.66 ± 0.33
	0.08	8.33 ± 0.33a
	0.09	8.66 ± 0.57a
$CdCl_2$ group	0.0016	5.66 ± 0.33
	0.02	8.33 ± 0.57a
	0.04	8.33 ± 0.33a
	0.06	8.33 ± 0.57a
$MnCl_2$ group	0.02	6.11 ± 0.33
	0.03	6.33 ± 0.35
	0.04	7.33 ± 0.56
	0.05	7.66 ± 0.59
$ZnCl_2$ group	0.02	5.66 ± 0.15
	0.04	5.69 ± 0.28
	0.06	6.12 ± 0.33
	0.08	6.33 ± 0.54



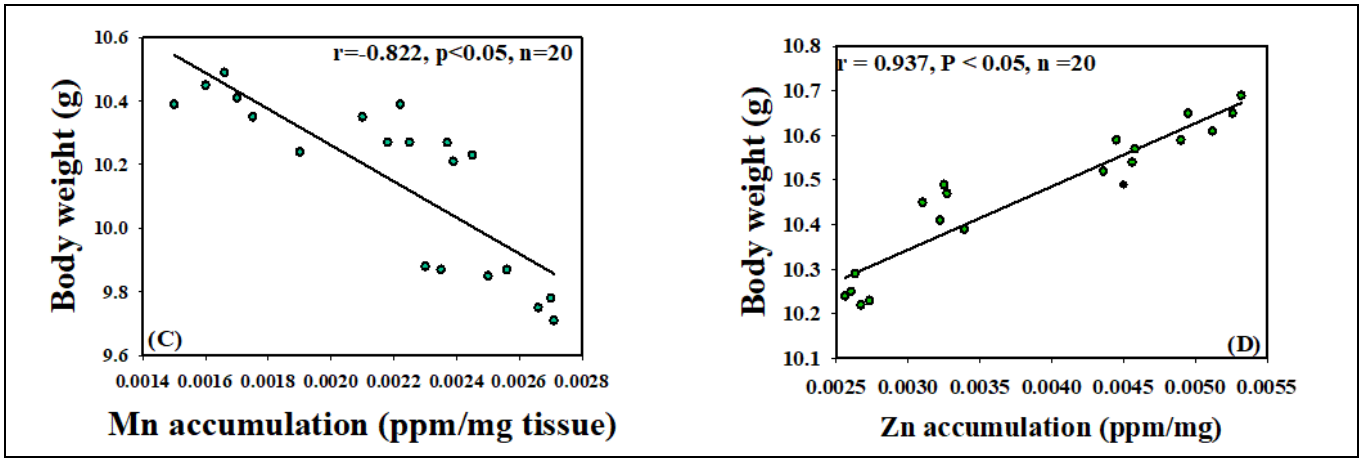


Fig 1: Correlations between body weight & metal accumulations in the pupa of *A. assamensis.* A, B, C & D represents effect of accumulated Cd, Pb, Mn & Zn on body weight on 3rd day. R implies correlation coefficient. Values differ significantly at P<0.05.

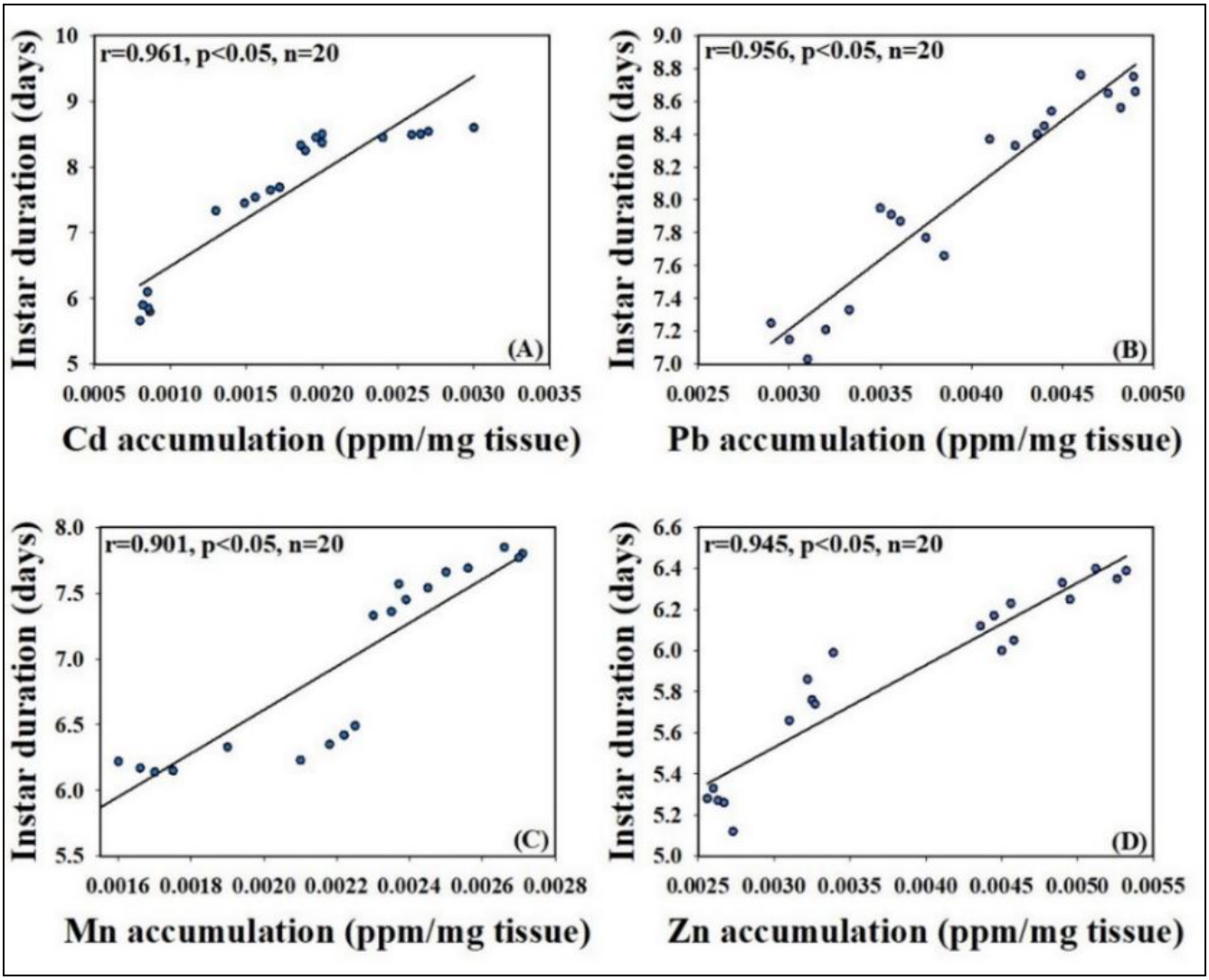


Fig 2: Correlations between instar duration & metal accumulations in the pupa of *A. assamensis.* A, B, C & D represents effect of accumulated Cd, Pb, Mn & Zn on body weight on 3rd day. R implies correlation coefficient. Values differ significantly at P<0.05.

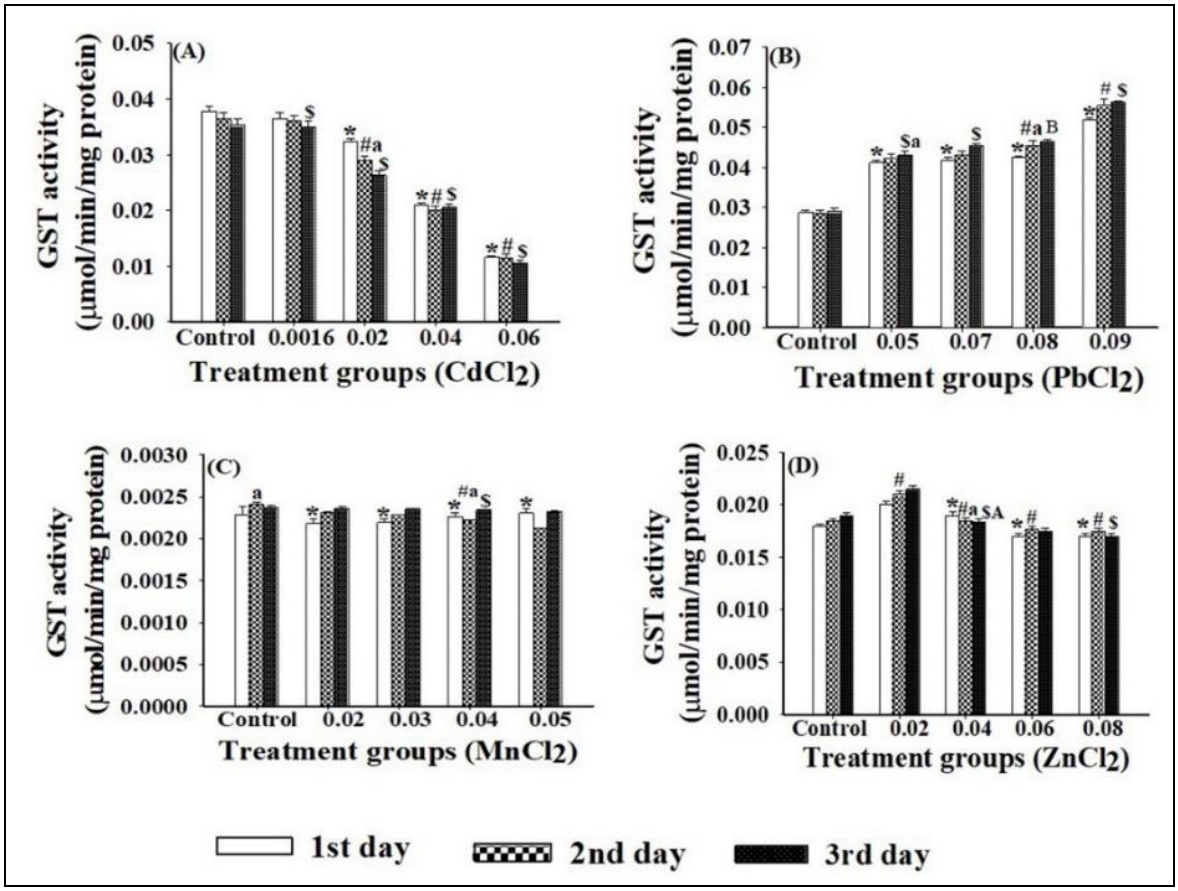


Fig 3: Glutathione-S-Transferase (GST) activity of *A. assamensis* after treatment. A, B, C and D represents effect of $CdCl_2$, $PbCl_2$, $MnCl_2$ and $ZnCl_2$ from 1st to 3rd day of exposure. Bars having superscripts "*", "#" and "$" represents the significant difference ($p<0.01$) across different doses of heavy metals from the control group respectively from 1st to 3rd day. Bars having superscripts of small and capital letters represents significant difference ($p<0.01$) within the each treatment group from 1st to 3rd day. Data were represented as mean ± SE (n=3).

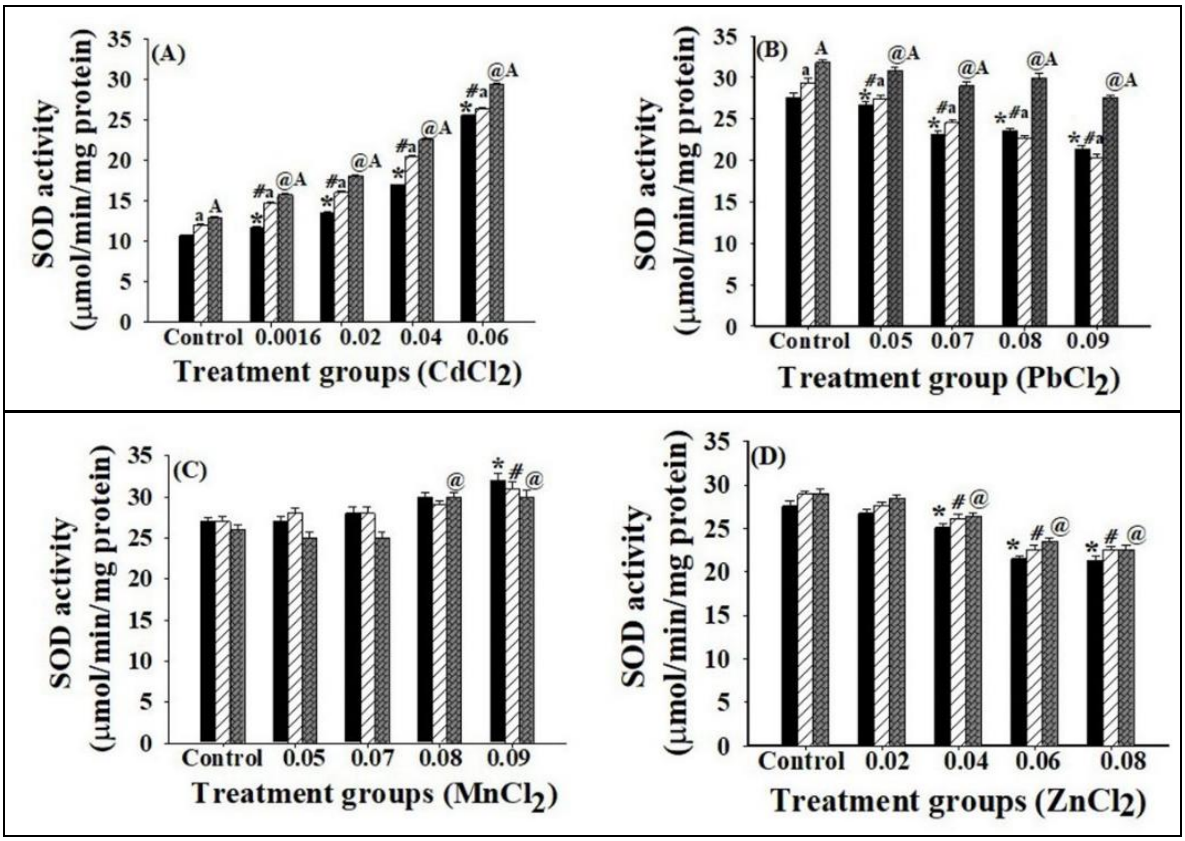


Fig 4: Superoxide dismutase (SOD) activity of *A. assamensis* after treatment. A, B, C and D represents effect of $CdCl_2$, $PbCl_2$, $MnCl_2$ and $ZnCl_2$ from 1st to 3rd day of exposure. Bars having superscripts "*", "#" and "$" represents the significant difference ($p<0.01$) across different doses of heavy metals from the control group respectively from 1st to 3rd day. Bars having superscripts of small and capital letters represents significant difference ($p<0.01$) within the each treatment group from 1st to 3rd day. Data were represented as mean ± SE (n=3).

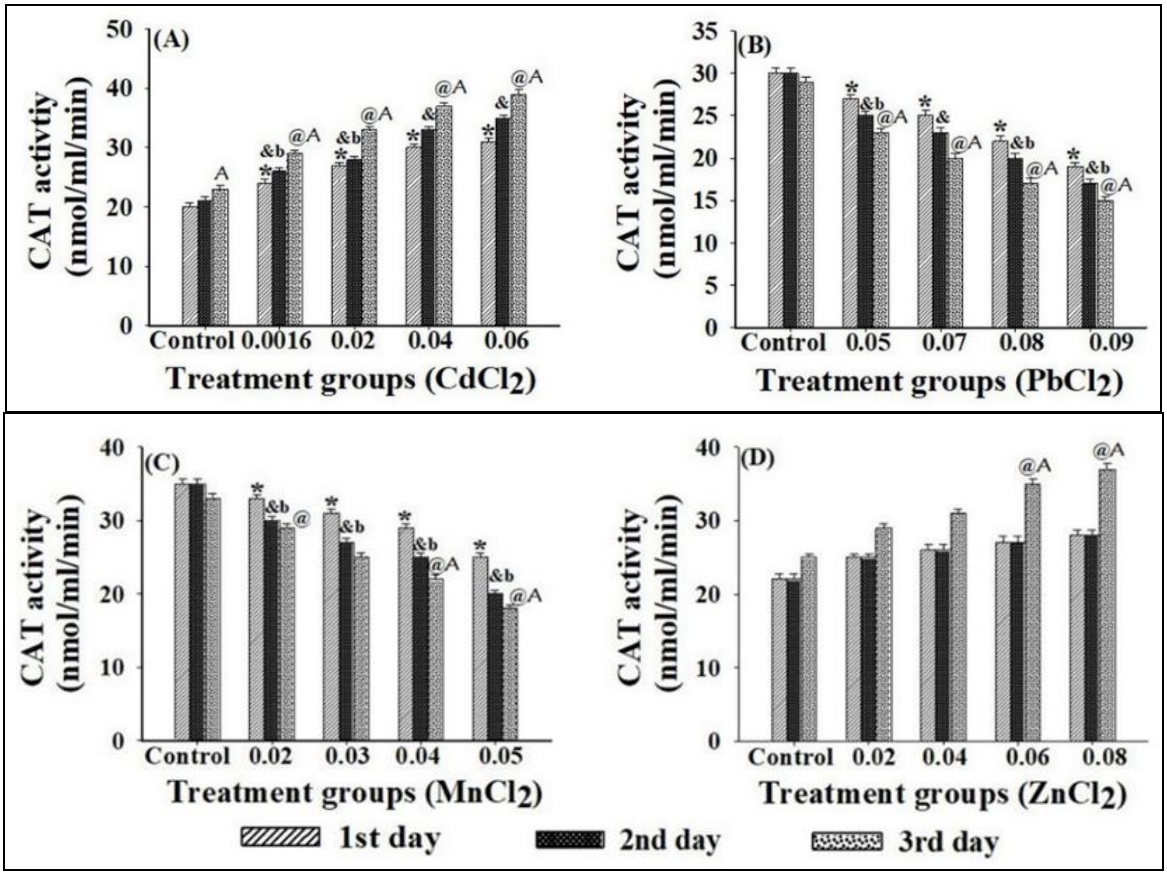


Fig 5: Catalase (CAT) activity of *A. assamensis a*fter treatment. A, B, C and D represents effect of $CdCl_2$, $PbCl_2$, $MnCl_2$ and $ZnCl_2$ from 1st to 3rd day of exposure. Bars having superscripts "*", "#" and "$" represents the significant difference ($p<0.01$) across different doses of heavy metals from the control group respectively from 1st to 3rd day. Bars having superscripts of small and capital letters represents significant difference ($p<0.01$) within the each treatment group from 1st to 3rd day. Data were represented as mean ± SE (n=3).

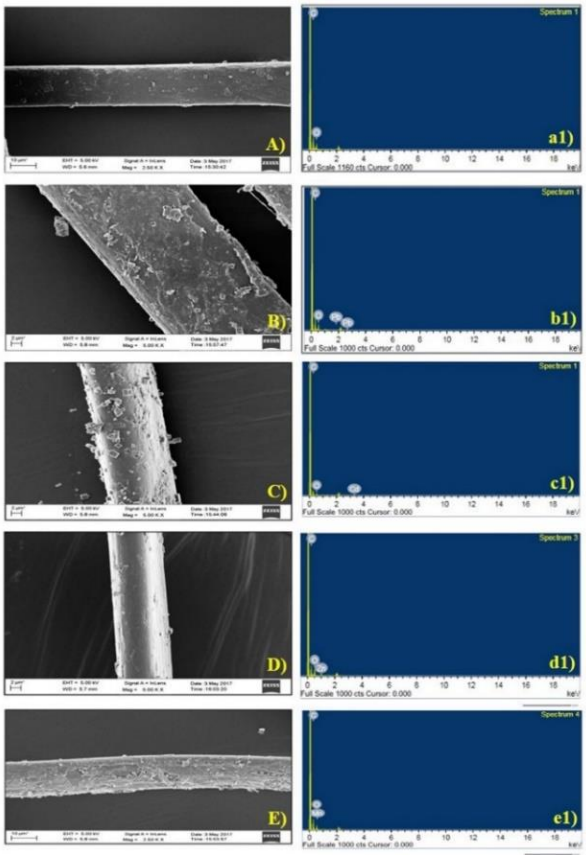


Fig 6: SEM and EDX image of *A. assamensis* silk after exposure to heavy metal dose. A, a1 represents images of control group silk thread. B, b1, C, c1, D, d1, E and e1 are the images after exposure to $CdCl_2$, $PbCl_2$, $MnCl_2$ and $ZnCl_2$ on silk thread.

5. Conclusion

This study is the first experiment to determine the antioxidant capacity and growth response of muga silkworm, found in North-East India to heavy metal pollutants. In summary, our results showed that antioxidant enzymes constituted effective defense mechanisms for *A. assamensis* larvae to resist toxicity originated from the accumulated Cd, Pb, Mn and Zn on *Machilus bombycina* leaves, but their defense level varied with heavy metal types and doses. Such information will further help in future to study the mechanisms underlying the effect of heavy metals on genetic level.

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7. Conflict of interest

The authors declare that they have no conflict of interest.

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