

1                   **Antioxidant enzymes as biomarkers of Cu and Pb exposures in ground spiders**

2                                   ***Lycosa terrestris* and *Pardosa birmanica***

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7                                   **Abstract**

8                   Heavy metal exposure promotes oxidative stress in terrestrial organisms, which they  
9                   counteract via activation of antioxidant biomarkers. The present study investigated the effects  
10                  of copper (Cu) and lead (Pb) on total antioxidant capacity (TAC) and antioxidant enzymes such  
11                  as Catalase (CAT), Glutathione reductase (GR), Superoxide dismutase (SOD) and Glutathione  
12                  peroxidase (GPX) in two spider species, namely *Lycosa terrestris* and *Pardosa birmanica*. The  
13                  spiders were exposed to Cu and Pb separately (10 ppm) or in combination (10 ppm each) via  
14                  different exposure routes (i.e. food and soil) for 10, 20 and 40 days. The results showed that  
15                  metal accumulation and antioxidant biomarker responses in spiders were metal- and species-  
16                  specific, and depended on the exposure route of the metals. The level of all antioxidant  
17                  biomarkers increased significantly with increasing exposure time and metal load in the bodies  
18                  of spiders via both uptake routes. The significant inhibition of TAC and enzyme activities was  
19                  only observed in single Pb treatment through soil exposure. In *L. terrestris*, the activities of  
20                  detoxification enzymes and TAC were significantly enhanced on single Cu exposure than Pb  
21                  via both exposure routes. However, in *P. birmanica* consistent variation among antioxidant  
22                  parameters were observed depending on the metal load and exposure routes. The combined  
23                  metal exposure caused more pronounced increase in the level of antioxidants compared to  
24                  single metal exposure in both species mainly via food exposure. These results suggest that the  
25                  antioxidant enzymes and TAC are sensitive to single and combined metal exposure via both  
26                  uptake routes. Furthermore, the antioxidant parameters can be used potential biomarkers of  
27                  oxidative stress induced by metal exposure and for monitoring environmental health using  
28                  spiders as bioindicators.

29                  **Keywords:** Ground spiders, Antioxidant system, Cu-Pb co-exposure, Uptake routes,  
30                  Bioindicator

## 32 1. Introduction

33 Environmental pollutants can cause disturbance at cellular and physiological levels, which  
34 in turn, produce harmful effects at higher levels of biological hierarchy such as population and  
35 communities (Regoli et al., 2014). Biomarkers can be utilized as early warning signals for one  
36 or several pollutants (Leomanni et al., 2016; Skaldina and Sorvari, 2017). Multiple biomarkers  
37 have been used as complementary tool in ecotoxicological monitoring to assess ecological risk  
38 of different environmental pollutants (Irizar et al., 2015; El-Gendy et al., 2019). In terrestrial  
39 organisms, several types of biomarkers, such as behavioral, physiological, immunological,  
40 hematological, enzymological can be used to evaluate harmful effect of pollutants (Zhiqun et  
41 al., 2017; Wilczek et al., 2018). Among these, the use of antioxidant enzyme defense system  
42 as biomarker of oxidative stress (OS) is widespread (Faria et al., 2010; Chainy et al., 2016).  
43 The imbalance in the formation of reactive oxygen species (ROS) in biological cells and its  
44 detoxification by antioxidant enzymes result in OS (Espín et al., 2016). Excessive accumulation  
45 of ROS and its intermediates inside the cells can produce oxidative cellular damage in the  
46 organism, such as oxidation of amino acid side chains, which disrupt protein structure and  
47 function, leading to inactivation of enzymes, damage of cell membrane, impairment of cell  
48 division and ultimately cell death (Gilbert, 1995; Davies and Shringarpure, 2006).

49 The ROS are chemically unstable and highly reactive free ions/radicals of oxygen  
50 naturally formed as by-products of cellular metabolism in redox reactions (Regoli and Giuliani,  
51 2014). The most common ROS includes single oxygen ( $O_2$ ) peroxides ( $O_2^{-2}$ ), superoxide anions  
52 ( $O_2^-$ ), hydroxyl radical (OH), hydroxyl ion ( $OH^-$ ) and hydrogen per-oxide ( $H_2O_2$ ). These  
53 oxyradicals differ in their cellular reactivity (Regoli and Giuliani, 2014; Dedeke et al., 2018).  
54 The exposure to xenobiotics such as pesticides, hydrocarbons and heavy metals increase the  
55 production of ROS in cells of organisms which in turn trigger antioxidant defense system to  
56 prevent oxidative damage in the exposed organisms (Leomanni et al., 2015; Gavrilović et al.,  
57 2017; Maity et al., 2018a). Antioxidants defense system of organisms comprises several  
58 enzymes, which function as scavenger of ROS intermediates that are produced inside cells. The  
59 main antioxidant enzymes are Glutathione reductase (GR), Catalase (CAT), Glutathione  
60 peroxidase (GPX) and Superoxide dismutase (SOD) (Gomes et al., 2015; Maity et al., 2018a).  
61 Initially, SOD protects cells from oxidative damage by catalyzing conversion of oxygen  
62 derivative superoxide anion ( $O_2^-$ ) into hydrogen peroxide ( $H_2O_2$ ) through dismutation.  
63 Subsequently, the activity of SOD is coordinated with  $H_2O_2$  reducing enzymes, such as CAT  
64 and GPX. These enzymes help in decomposition of  $H_2O_2$  into less toxic gaseous oxygen ( $O_2$ )

65 and water. The GPX also catalyzes the reduction of lipid hydroperoxides into alcohol through  
66 parallel oxidation of GSH (reduced glutathione) to GSSG (glutathione disulfide). The enhanced  
67 production of GPX results in accumulation of GSSG and depletion of GSH in the cells. As a  
68 result, GR becomes activated and catalyzes the reduction of oxidized GSSG back into GSH  
69 and helps in replenishment GSH into the system (Maity et al., 2018a). Thus, all of these  
70 antioxidant enzymes are crucial in maintaining homeostasis inside the cells. The total  
71 antioxidant capacity (TAC) measures the total antioxidative status of the organisms against  
72 xenobiotics and reflects the level of efficiency of antioxidants in organisms to scavenge toxic  
73 radical than separate measurement of each antioxidant (Franco et al., 2016).

74 In terrestrial environment, contamination by copper (Cu) and lead (Pb) is a major  
75 concern due to anthropogenic activities such as industrial waste, application of fertilizers,  
76 pesticides and sewage sludge in agricultural lands, waste water irrigation, atmospheric  
77 deposition and waste disposal (Wuana and Okieimen, 2011; Ali et al., 2015). Both metals are  
78 often found together in metal contaminated terrestrial areas (Wuana and Okieimen, 2011) and  
79 can be taken up by soil dwelling organisms via their food, water or dermal contact (Peijnenburg  
80 et al., 2012). Cu is a crucial metal for cell metabolism of aerobic organisms because it is a co-  
81 factor for several mitochondrial enzymes such as SOD, cytochrome P450 and terminal  
82 enzymes of the electron transport chain (Tchounwou et al., 2012; Tagliaferro et al., 2018). Cu  
83 is also a component of oxygen carrier respiratory protein “hemocyanin” in mollusks and  
84 arthropods (Burmester 2013; González et al., 2017). However, the high concentration of Cu  
85 inside cells mediate excessive generation of ROS, which overcome defense system of  
86 organisms and produce alterations in biological molecules, such as mitochondria, proteins,  
87 lipids and nucleic acid (Company et al., 2004; Xu et al., 2018). Pb is a pervasive element in the  
88 environment, which has no physiological role in the organisms (Yin et al., 2018). Previous  
89 studies shown that excessive intake of Pb can produce OS by inhibiting sulfhydryl (SH) group  
90 of several enzymes such as, GPX, CAT, GR, SOD and GST (Dai et al., 2012; Wang et al.,  
91 2015).

92 Among terrestrial invertebrates, spiders are frequently used in ecotoxicological studies  
93 to measure the effect of environmental changes (Yang et al., 2016). They are one of the most  
94 resistance invertebrate groups to metals and are relatively abundant in highly metal-polluted  
95 areas (Babczynska et al., 2012; Yang et al., 2016). Therefore, they are recognized as an ideal  
96 bioindicator species of environmental health. As secondary consumers, spiders are known to  
97 accumulate metals, such as Cu, Pb, Cd, Zn, specifically from their food and deposit them as

98 mineral granules in the midgut glands (Babczynska et al., 2011; Stalmach et al., 2015).  
99 Subsequently, metals are permanently stored in intracellular cells, digested or detoxified  
100 (Wilczek, 2017). Also, it is possible that metals can enter into spider's body via different  
101 routes, such as soil, water or contact with contaminated substrate (Chen et al., 2011; Erickson,  
102 2018).

103 In the present study, wolf spiders *Lycosa terrestris* and *Pardosa birmanica* were  
104 exposed to Cu and Pb separately and in combination via soil and food after different time  
105 intervals (10, 20 and 40 days), because enzymatic responses of organisms are time-dependent  
106 (Gomes et al., 2015; Maity et al., 2018a). The main aim of the present study was to evaluate  
107 the effectiveness of different antioxidant responses TAC, CAT, GPX, SOD and GR as potential  
108 biomarkers of Cu and Pb exposure in wolf spiders *Lycosa terrestris* and *Pardosa birmanica*.  
109 We hypothesized that (i) increase in metals accumulation will increase the activities of  
110 antioxidant enzymes (ii) Cu being an essential metal will induce less oxidative stress than Pb.  
111 (iii) Combined metals exposure will enhance the activities of antioxidant enzymes due to higher  
112 toxicity than Cu and Pb alone (iv) Both spiders' species show similar pattern of antioxidant  
113 activities as they belong to same family.

## 114 2. Material and Methods

### 115 2.1 Spiders Species

116 The present study was conducted on the fourth instar spiderlings of *L. terrestris* Butt et  
117 al., 2006 and *P. birmanica* Simon, 1884 (Lycosidae). Both species are active hunter and  
118 abundantly present in the fields of Punjab, Pakistan. Under natural condition, they mainly  
119 found running on the ground but *L. terrestris* also frequently visit foliage to capture their prey  
120 (Tahir et al., 2011). The members of both species are generalist predators and feed on  
121 Homoptera, Diptera, Hetroptera, Lepidoptera, Orthoptera and Araneae but in different  
122 proportions (Tahir and Butt, 2009). The total developmental duration from 2<sup>nd</sup> to 8<sup>th</sup> instars was  
123 155-160 days for both species.

124 For the experiment, the fourth instar spiderlings (n = 500) of each species were  
125 randomly collected from population reared on an artificial diet at Arachnology laboratory,  
126 Department of Zoology, University of the Punjab, Lahore, Pakistan. The time elapsed between  
127 each molt (i.e. 3<sup>rd</sup> to 4<sup>th</sup> instar) was used as diagnostic criterion for selection of specimens at  
128 their particular developmental stage. The spiders were kept at  $29 \pm 4$  °C,  $60 \pm 10$  relative  
129 humidity and 14h light: 10h dark photoperiod in separate plastic boxes with pierced caps (7.5

130 × 5.5 × 6 cm). A 2-cm thick artificial soil layer was placed over the bottom of plastic boxes.  
131 The artificial soil was prepared following recommendations of OECD (1984). The soil contents  
132 comprise air-dried mixture of 70% industrial sand with kaolin clay (20 %) and 10 % sphagnum  
133 peat. The soil pH was maintained at  $6.0 \pm 0.5$  with  $\text{CaCO}_3$  and moisture content at 50 % WHC  
134 (Water holding capacity). The artificial food was supplied *ad libitum* on a wax paper in the  
135 form of food-soaked cotton balls. The artificial food was made up with an equal mixture of  
136 dairy milk and soya milk (100 ml /100 ml) and one egg yolk as described previously (Amalin  
137 et al., 2001).

138

## 139 **2.2 Metal solutions**

140 Exposure concentration of copper (Cu) and lead (Pb) were chosen based on published  
141 data on bioaccumulation response of spider's species (Jung et al., 2005; Babczynska et al.,  
142 2011). The salts of  $\text{CuSO}_4 \times 5\text{H}_2\text{O}$  and  $\text{PbCl}_2$  (Sigma Aldrich, USA) were dissolved in distilled  
143 water to prepare 1 M stock solutions. Nominal metal concentrations (10 mM Cu, 10 mM of Pb  
144 and their mixture) were prepared by diluting primary stock solutions with distilled water.

## 145 **2.3 Food and soil contamination**

146 Spiderlings (n = 60 per treatment) of both species were exposed to uncontaminated, Cu  
147 contaminated, Pb contaminated or Cu + Pb contaminated artificial soil or food in separate  
148 experiments. The contaminated artificial soil or food was prepared by adding either water (in  
149 control) or aqueous solutions of metals in soil or food to make desired concentration of Cu (635  
150 mg/Kg) or Pb (2000 mg/Kg) and combination of both metals (Cu 635 mg/Kg + Pb 2000  
151 mg/Kg). The metal exposure experiments were performed for 40 days. The samples for metal  
152 quantification and enzymes analysis were collected after 10, 20 and 40 days.

## 153 **2.4 Metals Analysis**

154 Metals concentrations were measured as describe by Gouille et al. (2005) and Joy et al.  
155 (2015) by means of Inductively coupled plasma mass spectrometry (ICP-MS) of Thermo Fisher  
156 Scientific, USA. Ten spiders from each experimental group were randomly selected and freeze  
157 dried. Spiders were weighed ( $4.00 \pm 0.4$  mg) and acid digested singly in 3 ml of  $\text{HNO}_3$  (70%)  
158 and 2 ml  $\text{H}_2\text{O}_2$  (30%) with 3ml deionized water. After one-hour incubation, the solution was  
159 heated in a microwave oven at 140 °C for 10 minutes and at 55 °C for 20 minutes. The mixtures  
160 obtained after digestion were diluted with 6 ml distilled water. To check the precision and  
161 accuracy of the instrument certified reference material (CRM) bovine liver 1577c was used.

162 The percentage recovery of metal-spiked samples was more than 90% for both metals. Total  
163 metal concentrations were expressed as mg/kg dry matter.

## 164 **2.5 Enzyme Preparation and Assays**

165 After metal exposure, 10 spiders of each species were collected from different  
166 experimental as well as control groups after 10, 20 and 40 days and rinsed properly using  
167 distilled water to remove extra soil particles from their skin. Spiders were anaesthetized at 4  
168 °C for 2-3 minutes and homogenized singly in 1 ml of 0.05 M ice-cold homogenization  
169 Na<sub>2</sub>HPO<sub>4</sub>–KH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.4) in a prechilled mortar and pestle. The crude homogenate  
170 was collected in separated tubes and centrifuged for 10 minutes at 10,000 ×g to obtained clear  
171 supernatant, which was immediately used in different biochemical assays. During enzyme  
172 assays, supernatant was stored at 4°C.

## 173 **2.6 Total Protein Content quantification**

174 A colorimetric Coomassie Blue G-250 dye binding assay for quantification of protein  
175 in spider's homogenates was performed. as described previously (Bradford, 1976). A standard  
176 curve was generated using different dilutions of Bovine serum albumin (BSA). The OD was  
177 measured at 595 nm after incubation for 10 minutes at 37°C. The concentration of protein in  
178 unknown samples was quantified using standard curve of BSA.

## 179 **2.7 Biochemical Assays**

### 180 **2.7.1 Total antioxidant Capacity**

181 TAC was assayed following the protocol of Re et al., (1999). In assay mixture, the  
182 ability of antioxidants to reduce ABTS<sup>+</sup> radical cation (2,2'-azino-bis-(3-ethylbenzothiazoline-  
183 6-sulphonic acid)) was assessed. The reaction mixture was prepared by mixing 14 mM of  
184 ABTS (C<sub>18</sub>H<sub>18</sub>N<sub>4</sub>O<sub>6</sub>S<sub>4</sub>) solution with 7 mM of K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> in equal concentration and placed in  
185 dark for 16 hours. The subsequent blue-green solution was diluted up to 0.70 (OD) at 734 nm  
186 in PBS (pH 7.4). To start scavenging reaction, 10 ul of supernatant and 90 ul of ABTS solution  
187 was mixed and OD was measured after 5 minutes at 734 nm. Standard curve was constructed  
188 with different concentrations of Trolox to calculate TAC of unknown samples. The quantity of  
189 Trolox was expressed in mM/mg protein.

### 190 **2.7.2 Superoxide dismutase activity**

191 SOD activity was measured following standard protocol of Paoletti et al. (1986) based  
192 on decrease in OD value at 340 nm after the addition of NADPH in reaction. The assay mixture  
193 contains 10 mM Triethanolamine-diethanolamine (Tea-Dea) buffers, 50 ul supernatant, 7.5  
194 mM NADPH, mixture of 0.05 M MnCl<sub>2</sub> with 0.1 M EDTA, and 10 mM mercaptoethanol. The  
195 SOD activity was quantified in U/mg protein.

### 196 **2.7.3 Catalase**

197 The CAT activity was analyzed according to the method of (Clairborne, 1985). The  
198 reaction mixture comprised, 50 ul supernatant, 0.019 M H<sub>2</sub>O<sub>2</sub> in 0.05 M phosphate buffer of  
199 pH 7.0. The OD was read at 240 nm. The activity of CAT was quantified as decrease in  
200 absorbance due to depletion of substrate (H<sub>2</sub>O<sub>2</sub>) using 43.60 M<sup>-1</sup> cm<sup>-1</sup> extinction coefficient.

### 201 **2.7.4 Glutathione Peroxidase**

202 GPX assay was based on oxidation of NADPH (Mohandas et al., 1984). In reaction  
203 medium, oxidized glutathione use H<sub>2</sub>O<sub>2</sub> as a substrate and reduced to GSH. The 1.5 ml assay  
204 mixture comprises, 50 ul supernatant, 50 mM phosphate buffer (pH 7.0), 0.2 mM NADPH,  
205 0.1U/ml GR, 1 mM EDTA, 1 mM GSH, 1 mM NaN<sub>3</sub> and 0.25 mM H<sub>2</sub>O<sub>2</sub>. The OD was recorded  
206 at 340 nm. The GPX activity was determined using 6,220 M<sup>-1</sup> cm<sup>-1</sup> extinction coefficient and  
207 expressed in μm NADPH min<sup>-1</sup> mg<sup>-1</sup> protein.

### 208 **2.7.5 Glutathione Reductase**

209 The activity of GR was examined as according to the method of (Racker, 1955). The  
210 enzyme assay includes the mixture of 1 mM glutathione disulfide (GSSG), 1 mM EDTA, 0.1  
211 mM NADPH and 50 ul supernatant in phosphate buffer of pH 7.4. The decrease in NADPH  
212 level was read at 340 nm and quantified in μm min<sup>-1</sup> mg<sup>-1</sup> protein with 6,220 M<sup>-1</sup> cm<sup>-1</sup>  
213 extinction coefficient.

### 214 **2.7.6 Statistical analysis**

215 The metal concentrations and antioxidant parameters in spiders species were presented  
216 as mean ± standard error (SE). The normality of the data was calculated by using Kolmogorov–  
217 Smirnov test. One-way ANOVA following Tukey's test was employed to calculate differences  
218 between metal accumulation and variations in biomarker activities with exposure time. The  
219 biomarker parameters were analyzed using GLM (General linear model), in order to determine  
220 interaction between metal treatments, and exposure routes and time. The Pearson's correlation

221 was performed to test the strength of correlation among enzymatic biomarkers and with metal  
222 concentrations in spider's species. The difference in the enzymatic responses between spiders  
223 species was calculated by two-sample t-test. All statistical analysis was performed using  
224 Minitab 18 and  $P$ -values  $< 0.05$  were designated as significant.

### 225 **3. Results**

226 After 40 days, the cumulative mortality in *L. terrestris* ranges from 1-26 % and in *P.*  
227 *birmanica* 1-24% depending on the type of metal treatment. However, the average mortality in  
228 the control group was very low (0-2% *L. terrestris* and 0-6% in *P. birmanica*) in both species.

#### 229 **3.1 Metal accumulation**

230 The metal concentrations in artificial food, soil and spider's species is presented in  
231 Table 1 after subtracted from control groups. The bioaccumulation of Cu and Pb increased with  
232 exposure time in both species in either separate or combined metals treatments. In the metal  
233 mixture treatment, Cu accumulation decreased nearly two times as compared to single metal  
234 treatments via food in both spider's species ( $F = 74.83$ , d.f. = 1,  $P < 0.001$ , *L. terrestris*;  $F =$   
235  $75.02$ , d.f. = 1,  $P < 0.001$  *P. birmanica*). However, significant reduction in Pb uptake rate was  
236 observed in soil treatment ( $F = 43.47$ , d.f. = 1,  $P < 0.001$ , *L. terrestris*;  $F = 22.86$ , d.f. = 1,  $P <$   
237  $0.001$ , *P. birmanica*). In soil exposure assay, irrespective of metal's type and treatment, the  
238 mean metals concentrations were significantly higher in *L. terrestris* than *P. birmanica* ( $F =$   
239  $47.92$ ,  $P < 0.001$  for Cu) ( $F = 31.72$ ,  $P < 0.001$  for Pb) ( $F = 30.81$ ,  $P < 0.001$  for Mix-Cu) ( $F =$   
240  $8.27$ ,  $P = 0.006$  for Mix-Pb). However, both species accumulated almost similar amount of  
241 metals via food exposure in all treatments (Table 1).

#### 242 **3.2 Antioxidant parameters**

243 The activities of TAC, CAT, GPX, SOD and GR in *L. terrestris* and *P. birmanica* after  
244 exposure to Cu and Pb separately or in combination via food and soil at 10, 20 and 40 days are  
245 shown in Fig. 1. The enzymatic activities in control groups increased but not significantly with  
246 the increase in exposure time Fig. 1

##### 247 **3.2.1 Total antioxidant Capacity (TAC)**

248 The TAC (mM/mg protein) was significantly higher in all treatments as compared to  
249 control in both spider species (Fig. 1A, B; Table 2). TAC level varied significantly with respect  
250 to metal treatments, exposure routes, exposure time and their interaction in both species with

251 some exceptions. In *L. terrestris* interaction between exposure routes and time ( $P = 0.120$ ) and  
252 interaction of exposure routes, time and metal treatments ( $P = 0.189$ ) did not differ  
253 significantly. In *P. birmanica* TAC level did not differ among routes of exposure ( $P = 0.086$ )  
254 (Table 2). The highest level of TAC was recorded during combine metal treatment (Cu + Pb)  
255 through soil exposure, where it was more than four times higher in *L. terrestris* and five times  
256 in *P. birmanica* than in the control after 40 days of exposure (Fig. 1A, B; Table 2). A significant  
257 positive correlation between TAC and total metals concentrations was observed in all  
258 treatments except in Pb treatment via soil exposure in both species (Table 3). Among both  
259 species significant difference in TAC level was only recorded in single Cu ( $t = 6.78, P < 0.001$ )  
260 and Pb treatments ( $t = 2.29, P = 0.026$ ) via soil and food exposures, respectively.

### 261 3.2.2 Superoxide dismutase (SOD)

262 The SOD (U/mg protein) level of both species increased significantly with increasing  
263 metals concentration (Cu or Pb) in body of spiders expect in the case of Pb exposure via soil  
264 (Fig. 2A, B; Table 3). In Pb treatment via soil, the SOD activity was two times higher than  
265 control group until day 10 thereafter, significantly decreased with increasing Pb concentration  
266 ( $r = -0.876, P < 0.001$  *L. terrestris*;  $r = -0.723, P < 0.001$  *P. birmanica*) in both spider species.  
267 The results of GLM indicated that all three tested factors (i.e. metal treatments, exposure routes  
268 and time individually and their interactions) were significantly different in both species.  
269 However, in *L. terrestris* SOD level did not differ among exposure routes (Table 2). Both  
270 species showed almost similar pattern of SOD activity in all treated groups with the exception  
271 of single Cu ( $t = 2.85, P = 0.006$ ) and Cu + Pb treatments ( $t = 3.44, P = 0.001$ ) via soil  
272 exposures.

### 273 3.2.4 Catalase (CAT)

274 CAT ( $\mu\text{M}/\text{min}/\text{mg}$  protein) level fluctuated remarkably in both spider species under  
275 different experimental conditions (Fig. 3A, B; Table 2). In *L. terrestris* the highest CAT activity  
276 was recorded during combined metal exposure via food, which was about 14-times higher than  
277 control. However, CAT level during combined metal treatment via soil was nearly two times  
278 lower than combined metal exposure via food (Fig. 3A). In *P. birmanica*, CAT induction was  
279 highest during single Pb exposure via food (Fig. 3B). A strong positive correlation of CAT  
280 activities with metal concentration of body was recorded in both spiders species except for Pb  
281 exposure via soil ( $r = -0.698, P < 0.001$  *L. terrestris*;  $r = -0.739, P < 0.001$  *P. birmanica*;

282 Table 3). Furthermore, the level of CAT was significantly different ( $P < 0.001$ ) among both  
283 species in all treated groups.

### 284 3.2.5 Glutathione peroxidase (GPX)

285 The GPX ( $\mu\text{M}/\text{min}/\text{mg}$  protein) activities was significantly increased with time in all  
286 experimental groups compared to control (Fig. 4A, B; Table 2). The strong positive correlation  
287 between internal metal concentration and GPX level was observed in both spiders species in  
288 all treatments except during single Pb treatment via soil, where GPX activity decreased but not  
289 significantly in *P. birmanica* ( $r = -0.489$ ,  $P = 0.006$  for *L. terrestris*;  $r = -0.039$ ,  $P = 0.838$  for  
290 *P. birmanica*; Table 3). The GPX level was nearly two times higher during combined metal  
291 exposure via soil than via food in both species (Fig. 4A, B). The GPX activity was significantly  
292 higher in *P. birmanica* in all treated groups than *L. terrestris* except during single Cu ( $t = 1.10$ ,  
293  $P = 0.278$ ) and Cu + Pb exposures ( $t = 1.02$ ,  $P = 0.311$ ) via soil.

### 294 3.2.6 Glutathione reductase (GR)

295 The GR induction by metals exposure followed nearly the similar trend as that of GPX  
296 in both spider species (Fig. 5A, B; Table. 2). In *L. terrestris*, the maximum GR activity was  
297 recorded during combined metal exposure via food while in *P. birmanica* level of GR was  
298 highest during Pb-only exposure via food (Fig. 5A, B). A significant negative correlation  
299 between GR level and Pb concentration via soil exposure in both spiders species was detected  
300 ( $r = -0.698$ ,  $P < 0.001$  *L. terrestris*;  $r = -0.739$ ,  $P = 0.000$  *P. birmanica*; Table. 3). The  
301 induction of GR was also significantly different in both spider's species among all treated  
302 groups, but Cu ( $t = 1.62$ ,  $P = 0.112$ ) and Cu + Pb exposures ( $t = 1.92$   $P = 0.060$ ) via food.

### 303 3.3 Correlation between TAC and antioxidants enzymes

304 Here, TAC level showed high correlation with different enzymes activities (SOD, CAT,  
305 GPX, GR) depending on metal treatments and its exposure routes (Table 3). The correlation  
306 analysis among different antioxidant enzymes revealed that not only metal concentration, but  
307 also metal type and their exposure routes were important factors in controlling biochemical  
308 responses of spiders.

## 309 4. Discussion

310 The natural and anthropogenic activities can lead to deposition of various pollutants,  
311 such as heavy metals into the ecosystem (Wu et al., 2012). As a result, soil organisms can be

312 simultaneously exposed to multiple metals instead of a single metal, via different uptake routes  
313 such as food, pore water, dermal contact or respiration (Peijnenburg et al., 2012). Therefore,  
314 laboratory studies relying on single metal exposure through dietary route do not offer a realistic  
315 approach towards understanding metal toxicity. Here, the uptake of Cu and Pb from food or  
316 soil during separate and combined metal exposures in the 4<sup>th</sup> instar spiderling of *L. terrestris*  
317 and *P. birmanica* was measured after 10, 20 and 40 days. The 4<sup>th</sup> instar spiderlings were used  
318 due to increase in body mass and developmental time whereas decrease in mortality at this life  
319 stage. The main reason for using the long exposure duration (40 days) was to increase the  
320 chance of metals uptake from food as well as from soil and their effect on antioxidant activities.  
321 Exposure time has a significant effect on accumulation of metals, its toxicity and antioxidant  
322 responses of spiders. In the present study, once the 3<sup>th</sup> instar spiderlings molted, they were  
323 exposed to metal contaminated food or soil. During 20 days of exposure, the body size of  
324 spiderlings increased considerably either in control or metal treated group but they were still at  
325 4<sup>th</sup> instar stage. The spiderlings in the control groups molted after 22-24 days and in metal  
326 treated groups after 31-36 days depending on the type of metal treatment. At day 40, all  
327 spiderlings (either in control or metal treated groups) were at 5<sup>th</sup> instar stage. In 40 days,  
328 spiderlings molted one time so we can observe changes in antioxidant activities both in 4<sup>th</sup> and  
329 5<sup>th</sup> instar spiderling during this time duration.

330 We showed that both spiders species accumulated significant amount of metals in time-  
331 dependent manner and the level of metals in the spider body depended on the type of metal,  
332 duration of exposure, uptake route and spiders species. Both species accumulated highest  
333 amount of Cu via their food, whereas Pb accumulation was highest during soil exposure. Rybak  
334 et al. (2019) reported that *Eratigena atrica* (Agelenidae) accumulated 2 to 7 times higher  
335 concentration of Cu in their bodies compared to Pb by dietary uptake. Yang et al. (2016)  
336 clustered Lycosidae and Agelenidae in the same group because both families showed similar  
337 pattern of metal accumulation. In combined metal exposure, the interaction between Cu and  
338 Pb at uptake sites significantly influence the absorption of both metals and hence accumulation.  
339 The Cu concentration was significantly reduced in the presence of Pb during food exposure  
340 while Pb accumulation was decreased during combined metals exposure via soil. This  
341 antagonistic interaction between Cu and Pb might be due to the common uptake route i.e. Na<sup>+</sup>  
342 channels for their entry into cells. The antagonistic interaction between Cu and Pb was also  
343 reported in *Scenedesmus vacuolatus* (green algae) Topperwien et al. (2007) and in  
344 *Chlamydomonas reinhardtii* (Unicellular algae) Lavoie et al. (2012). The present study also

345 showed that *L. terrestris* accumulated high concentration of both metals via soil exposure under  
346 similar experimental conditions. These data revealed that species-specific difference in metal  
347 accumulation exists. The high accumulation of metals from soil in *L. terrestris* as compared to  
348 *P. birmanica* may be attributable to the difference in their walking activity. The *L. terrestris* is  
349 more active than *P. birmanica* (Tahir et al., 2011). So, it may be possible that *L. terrestris*  
350 accumulates more metals from contaminated soil as compared to *P. birmanica* due to their  
351 walking activity and increased contact with the soil surface.

352 The measurement of total contamination level in organisms as a result of exposure to  
353 toxicants provides insufficient information regarding potential harmful effects of the  
354 contaminants to biota (Binelli et al., 2010). The development of suitable biological tools found  
355 in biological indicator species namely “biomarkers” has been widely used in risk assessment  
356 studies to measure the effect of contaminants in the exposed organisms. In our study, the  
357 variations in the level of antioxidant enzymes such as GR, SOD, GPX, CAT and TAC were  
358 used as biomarkers of Cu and Pb, in lycosids spiders *L. terrestris* and *P. birmanica* to evaluate  
359 their efficacy as biological tool for predicting the metal’s impact in terrestrial system. Both Cu  
360 and Pb are known to boost oxidative stress in organisms and exhibit distinct effects on  
361 antioxidant enzymes due to their different chemical reactivity (Nikolic et al., 2016). Cu is a  
362 redox active metal which induce oxidative stress by Fenton-like reactions, whereas Pb being  
363 redox inactive metal binding to sulfhydryl groups of proteins and inhibits enzyme functions  
364 (Ercal et al., 2001; Nikolic et al., 2016).

365 Previous studies have reported variable responses of antioxidants enzymes toward  
366 metal exposure. The enzyme level may increase, decrease or remain unchanged depending on  
367 various factors, such as metal type and concentration, model organism tested, exposure  
368 duration and experimental conditions (Zhang et al., 2010b; Yousef et al., 2019). In the  
369 examined spiders species, strong and significant increase in enzymatic parameters (SOD, CAT,  
370 GPX, GR) over time was observed under Cu, Pb and Cu + Pb exposures via both routes (food  
371 and soil) except for Pb exposure in soil. The high induction of antioxidant enzymes in response  
372 to metal accumulation showed that these enzymes possess an enhanced capability to scavenge  
373 ROS radical and protected organisms from oxidative damage (Wang et al., 2018). In contrast,  
374 during Pb-only exposure through soil, the level of enzymes significantly decreased with time  
375 and negative correlation with metal concentration was detected. This reduction in enzymatic  
376 activities might be due to high accumulation of Pb from soil compared to food (Table 1).

377 Enzyme inactivation prevails at high metal load due to excessive production of ROS, which  
378 causes inhibition of enzymatic activities (Franco et al., 2006; Maity et al., 2008).

379 The SOD is a crucial antioxidant enzyme that prevents cells from lipid peroxidation by  
380 catalyzing conversion of super-oxide radicals ( $O_2^-$ ) into  $H_2O_2$  and  $O_2$  (Wu et al., 2011). We  
381 found that metal accumulation significantly promoted SOD activities over time in both spider  
382 species. Previous studies also reported enhanced production of SOD in response to heavy metal  
383 exposure in spiders and other invertebrates. For instance, spider species including *Linyphia*  
384 *triangularis* (Linyphiidae), *Xerolycosa nemoralis* (Lycosidae), *Pardosa lugubris* (Lycosidae)  
385 collected from different metal-contaminated sites showed high SOD activity compared to  
386 reference sites (Wilczek and Migula, 1996; Wilczek, 2005; Wilczek et al., 2013). In another  
387 lycosid, *Pardosa pseudoannulata*, the level of SOD increased significantly after exposure to  
388 low dose of Cd in their drinking water (Li et al., 2016). However, gradual depletion of SOD  
389 activity has been reported in *Pirata subpiraticus* collected from metal-polluted sites and on  
390 dietary Cd exposure under laboratory conditions (Zhang et al., 2010a). These studies revealed  
391 that species-specific differences in antioxidant responses exist and even closely related species  
392 respond differently to oxidative stress triggered by metal contamination. Moreover, in our  
393 study the SOD level significantly reduced during single Pb exposure through soil probably due  
394 to high accumulation of Pb from soil as compared to food. A previous study has shown that the  
395 SOD activity in *Daphnia magna* was stimulated at low concentration of Pb, but inhibited at  
396 higher concentration (Kim et al., 2018). Furthermore, the assessment of antioxidant activities  
397 is generally performed by analysis of single metal only. To the best of our knowledge, there is  
398 very little information available about the potential effects of metal mixture on the antioxidant  
399 enzymes of the soil invertebrates.

400 We also found that the SOD activity was higher in response to Cu-only exposure than  
401 during Pb-only exposure via both routes. The highest SOD induction was observed during  
402 mixture assay in both species. Qian et al. (2011) reported that the activity of SOD in *Chlorella*  
403 *vulgaris* (green algae) increased almost five-folds following single Cu exposure and 5-9 folds  
404 after combined exposure to Cu and Cd depending on metal concentration. However, single Cd  
405 exposure did not significantly increase SOD activity. In the earthworm *Eisenia fetida*, the SOD  
406 level was significantly increased during single Pb exposure, but reduced significantly after  
407 simultaneous exposure of Pb with decabromodiphenyl ether (BDE209) (Zhang et al., 2015).  
408 These results suggest that SOD level in organisms is contaminant-specific and concentration-  
409 dependent.

410 Catalase is also an important antioxidant enzyme which protects biological cells from  
411 oxidative damage by reducing oxyradical ( $H_2O_2$ ) into less toxic forms (Regoli et al., 2011). In  
412 our study, the significant increase in CAT level with the increase in metal concentrations has  
413 been observed in all experimental groups in both spider species except during Pb-only exposure  
414 through soil. Wilczek (2005) and Wilczek et al. (2008) reported similar response to CAT  
415 induction in different spiders species collected from metal-polluted areas. Stalmach et al.  
416 (2015) showed the highest CAT activity during starvation period in *Xerolycosa nemoralis*  
417 (Lycosidae) compared to different concentrations of dimethoate. In the *Helix aspersa* (land  
418 snail), CAT level significantly increased after exposure to Cu, Pb and Zn pollution (Abdel-  
419 Halim et al., 2013). Likewise, in contrast to SOD activity, the CAT level in our study was  
420 higher during single Pb exposure via food compared to Cu exposure via both routes (food or  
421 soil). During combined metal exposure, the CAT level was nearly similar via both routes in *P.*  
422 *birmanica*. However, in *L. terrestris*, CAT values were significantly higher during food  
423 exposure as compared to soil. Mleiki et al. (2015) reported enhanced CAT level in foot and  
424 digestive glands of *Cantareus apertus* (garden snail) subjected to Cu, Cd, and their combination  
425 via artificial food. Another study on *Macrobrachium nipponense* prawns reported that the  
426 activity of CAT first increased significantly on exposure to low doses of Pb but subsequently  
427 decreased with increasing Pb concentration. This suggests that high concentration of lead can  
428 cause inhibition of enzyme function (Ding et al., 2019). The present study also demonstrated  
429 that the CAT activity is species-specific and showed high sensitivity towards metals in  
430 maintaining the balance between oxidative stress and antioxidant defenses.

431 GPX is a cytosolic enzyme that protects the cells from oxidative damage by catalyzing  
432 hydrogen peroxide conversion into water and oxygen like catalase. GPX also catalyzes the  
433 conversion of lipid hydro-peroxides into alcohol by oxidizing reduced glutathione (GSH) into  
434 glutathione disulfide (GSSG) (Regoli and Giuliani, 2014). It is intriguing that the GPX activity  
435 was increased in a time-dependent manner as metal accumulation increased in the body of the  
436 spiders. Both metals significantly induced GPX activity and the highest level was observed  
437 during metal mixture via food. Wilczek et al. (2004) reported high activity of both selenium-  
438 dependent and independent glutathione peroxidases in *Agelena labyrinthica* (Agelenidae)  
439 compared to *Pardosa lugubris* (Lycosidae) collected from areas heavily polluted with Pb and  
440 Zn. Similarly, the GPX level was higher in *Theba pisana* (land snail) collected from metal-  
441 contaminated sites compared to the reference site, and GPX activity also correlated positively  
442 with metal concentrations (Zn, Cu, Pb and Cd) in snails (Radwan et al., 2010). In *Acipenser*

443 *ruthenus* (sperm) the GPX activity was significantly reduced during exposure to high  
444 concentration of Cr and Cr + Cd compared to Cd alone (Li et al., 2010). The present study  
445 showed that the high GPX activity together with CAT involved in scavenging of ROS and  
446 defense against OS induced by Cu and Pb exposure in these species.

447 The GR catalyze the reduction of glutathione disulfide (GSSG) into GSH to revive GSH  
448 pool in biological cell and comprise an important indicator of redox state (Ecimovic et al.,  
449 2018). The activity of GR increased with increasing exposure time in present study. In *L.*  
450 *terrestris* the highest GR level was detected during metal combined exposure via food while in  
451 *P. birmanica*, marked GR stimulation was noted in Pb-only exposure via food and GR activities  
452 was also positively correlated with metal concentrations in both spider species except during  
453 Pb exposure via soil. Stalmach et al. (2015) showed that the GR activity was highest in  
454 *Xerolycosa nemoralis* (Lycosidae) during high dose dimethoate exposure, however starvation  
455 had no effect on GR activity. In *Aporrectodea caliginosa* earthworms, the GR activity was  
456 positively correlated with accumulated metals concentration similar to GPX and GST (Maity  
457 et al., 2018b). Mircic et al. (2013) reported that the GR activity in *Lymantria dispar* following  
458 Cd exposure via artificial diet depends on their developmental stage. It was lower than control  
459 in third instar but increased significantly in sixth instar with increase in Cd accumulation. In  
460 the present study, the significant induction of GR is consistent with other antioxidants (GPX,  
461 CAT and SOD), indicating the existence of OS due to metal exposure in spiders.

462 The total antioxidant capacity (TAC) is useful tool to assess overall defense strategy of  
463 organisms against oxidative stress as a substitute of traditional antioxidant defenses (Cossi et  
464 al., 2018). In our study, TAC significantly increased in a time-dependent manner in all treated  
465 groups and the highest TAC was recorded during combine metals treatment via soil in both  
466 species. However, TAC level significantly decreased and negatively correlated with metal  
467 accumulation during Pb exposure via soil. A significant increase of TAC level was also  
468 observed in the hemolymph of *Xerolycosa nemoralis* (Lycosidae) after exposure to dimethoate  
469 and starvation as stress factors (Stalmach et al., 2015). In the gills of *Mytilus galloprovincialis*  
470 mussel exposed to Cu, Pb and Cd separately or in combination, TAC level increased non-  
471 significantly during all treatment (Pb, Cd or Cu + Pb + Cd) except during single Cu exposure  
472 (Franco et al., 2016). A non-significant increase in TAC values has been reported in *Mytilus*  
473 *galloprovincialis* after exposure to binary mixtures of Cu, Pb and Cd (Franco-Martinez et al.,  
474 2016). In *Eisenia fetida* earthworms, TAC level was highest during single Pb exposure, but the  
475 presence of decabromodiphenyl ether (BDE209) caused increase in Pb toxicity by inhibiting

476 TAC level (Liu et al., 2015; Zhang et al., 2015). These studies revealed that TAC was not only  
477 species-specific, but also contaminant-specific.

## 478 **5. Conclusion**

479 The present study demonstrated that the combined and separate exposure of spiders to Cu  
480 and Pb via both uptake routes (food and soil) induced significant changes in TAC and  
481 antioxidant activities in a time-dependent manner. The Cu exposure induced higher activities  
482 of antioxidant parameter while high accumulation of Pb caused inhibition in antioxidant  
483 activities, reflecting the impairment of the antioxidant defense system. The combined exposure  
484 of Cu and Pb exhibited enhanced responses of antioxidant parameters from those exposed to  
485 Cu and Pb alone. Both spiders' species shared the similar trend of antioxidant responses but  
486 showed some variation in their sensitivity corresponding to metal stress. The antioxidant  
487 activities showed positive correlation with metal body burden. Consequently, the antioxidant  
488 parameters can be applied as potential biomarkers for the assessment and monitoring of  
489 environmental pollution.

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761 Table 1. Quantity of Cu and Pb in artificial soil, food and spider's species after different time periods. The  
 762 treatments include, CuF (copper in food), CuS (copper in soil), PbF (lead in food), PbS (lead in soil), Mix-CuF  
 763 (copper in food during combine exposure) and Mix-CuS (copper in soil during combine exposure), Mix-PbF (lead  
 764 in food during combine exposure) and Mix-PbS (lead in soil during combine exposure).

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Test groups	Metals concentration mg/Kg Dry weight							
	CuF	CuS	PbF	PbS	Mix-CuF	Mix-CuS	Mix- PbF	Mix-PbS
Nominal	635	635	2000	2000	635	635	2000	2000
Measured	628.22 ± 2.20	629.45 ± 2.38	1934 ± 6.34	1891.40 ± 2.58	625.48 ± 3.19	628.18 ± 2.37	1955.50 ± 3.23	1884 ± 3.07
<i>Lycosa terrestris</i>								
10-days	29.30 ± 1.98 <sup>c</sup>	18.56 ± 2.04 <sup>c</sup>	6.608 ± 0.67 <sup>b</sup>	11.13 ± 1.24 <sup>c</sup>	6.54 ± 0.74 <sup>c</sup>	22.65 ± 2.30 <sup>c</sup>	4.84 ± 0.62 <sup>c</sup>	8.21 ± 1.02 <sup>c</sup>
20-days	71.44 ± 6.53 <sup>b</sup>	62.33 ± 5.92 <sup>b</sup>	16.20 ± 2.01 <sup>b</sup>	49.66 ± 4.95 <sup>b</sup>	32.59 ± 2.97 <sup>b</sup>	70.62 ± 6.20 <sup>b</sup>	14.75 ± 1.44 <sup>b</sup>	25.02 ± 2.20 <sup>b</sup>
40-days	135.50 ± 10.30 <sup>a</sup>	140.63 ± 9.06 <sup>a</sup>	42.58 ± 4.64 <sup>a</sup>	133.4 ± 9.48 <sup>a</sup>	73.38 ± 6.54 <sup>a</sup>	146.49 ± 8.15 <sup>a</sup>	36.41 ± 3.38 <sup>a</sup>	72.26 ± 7.69 <sup>a</sup>
<i>Pardosa birmanica</i>								
10-days	22.09 ± 2.81 <sup>c</sup>	20.25 ± 2.46 <sup>b</sup>	5.37 ± 0.84 <sup>c</sup>	12.57 ± 1.45 <sup>c</sup>	18.72 ± 2.43 <sup>c</sup>	29.59 ± 3.31 <sup>c</sup>	7.14 ± 1.04 <sup>b</sup>	11.78 ± 1.39 <sup>b</sup>
20-days	78.11 ± 7.73 <sup>b</sup>	36.44 ± 4.73 <sup>b</sup>	15.55 ± 1.38 <sup>b</sup>	33.27 ± 4.26 <sup>b</sup>	36.40 ± 4.95 <sup>b</sup>	51.46 ± 5.42 <sup>b</sup>	18.02 ± 1.80 <sup>b</sup>	19.74 ± 1.86 <sup>b</sup>
40-days	161.89 ± 9.65 <sup>a</sup>	69.64 ± 6.27 <sup>a</sup>	41.21 ± 3.33 <sup>a</sup>	74.86 ± 5.83 <sup>a</sup>	77.67 ± 5.64 <sup>a</sup>	82.98 ± 5.96 <sup>a</sup>	53.63 ± 5.41 <sup>a</sup>	46.79 ± 4.35 <sup>a</sup>

766 Data is represented in mean ± sem. Mean with different superscripts letters (a, b, c) letters show significant  
 767 difference between different exposure duration. (One-way ANOVA with Tukey's test at  $P < 0.05$ ).

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795 Table 2 Results of GLM (General linear model) on biochemical parameters (TAC, SOD, CAT, GPX, GR) in  
 796 spiders species exposed to Cu and Pb separately or in combination (treatments) via different routes (food, soil)  
 797 for 10, 20, and 40 days.

Biochemical parameters	<i>Lycosa terrestris</i>				<i>Pardosa birmanica</i>			
	Source of variations	DF	F	P	Source of variations	DF	F	P
TAC	Treatments	2	69.13	0.000	Treatments	2	120.16	0.000
	Routes	1	50.14	0.000	Routes	1	0.030	0.086
	Days	2	37.96	0.000	Days	2	55.56	0.000
	Treatments × Routes	2	7.87	0.001	Treatments × Routes	2	7.16	0.001
	Treatments × Days	4	10.31	0.000	Treatments × Days	4	8.32	0.000
	Routes × Days	2	2.15	0.120	Routes × Days	2	3.66	0.028
	Treatments × Routes × Days	4	1.55	0.189	Treatments × Routes × Days	4	2.87	0.025
SOD	Treatments	2	503.50	0.000	Treatments	2	236.37	0.000
	Routes	1	0.630	0.430	Routes	1	11.32	0.001
	Days	2	430.52	0.000	Days	2	282.43	0.000
	Treatments × Routes	2	82.28	0.000	Treatments × Routes	2	6.18	0.003
	Treatments × Days	4	82.23	0.000	Treatments × Days	4	38.42	0.000
	Routes × Days	2	60.46	0.000	Routes × Days	2	32.04	0.000
	Treatments × Routes × Days	4	56.26	0.000	Treatments × Routes × Days	4	21.81	0.000
CAT	Treatments	2	213.07	0.000	Treatments	2	45.42	0.000
	Routes	1	280.38	0.000	Routes	1	47.77	0.000
	Days	2	322.74	0.000	Days	2	287.92	0.000
	Treatments × Routes	2	24.48	0.000	Treatments × Routes	2	56.71	0.000
	Treatments × Days	4	18.10	0.000	Treatments × Days	4	9.93	0.000
	Routes × Days	2	73.38	0.000	Routes × Days	2	133.53	0.000
	Treatments × Routes × Days	4	47.02	0.000	Treatments × Routes × Days	4	54.67	0.000
GPX	Treatments	2	3.65	0.000	Treatments	2	5.14	0.007
	Routes	1	18.70	0.000	Routes	1	537.33	0.000
	Days	2	270.71	0.000	Days	2	150.8	0.000
	Treatments × Routes	2	55.46	0.000	Treatments × Routes	2	153.23	0.000
	Treatments × Days	4	33.71	0.000	Treatments × Days	4	3.63	0.007
	Routes × Days	2	20.82	0.000	Routes × Days	2	44.77	0.000
	Treatments × Routes × Days	4	7.09	0.000	Treatments × Routes × Days	4	7.13	0.000
GR	Treatments	2	163.24	0.000	Treatments	2	44.93	0.000
	Routes	1	53.16	0.000	Routes	1	225.81	0.000
	Days	2	176.57	0.000	Days	2	232.04	0.000
	Treatments × Routes	2	116.34	0.000	Treatments × Routes	2	150.96	0.000
	Treatments × Days	4	16.51	0.000	Treatments × Days	4	5.69	0.000
	Routes × Days	2	31.32	0.000	Routes × Days	2	71.85	0.000
	Treatments × Routes × Days	4	6.37	0.000	Treatments × Routes × Days	4	36.03	0.000

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808 Table 3 Pearson's correlation between biochemical responses (TAC, SOD, CAT, GPX, GR) and metal  
 809 concentration in spider's species exposed to Cu, Pb or their combination through food or soil for 10, 20 and 40  
 810 days. Asterisk (\*) indicates significant values at  $P < 0.05$ .

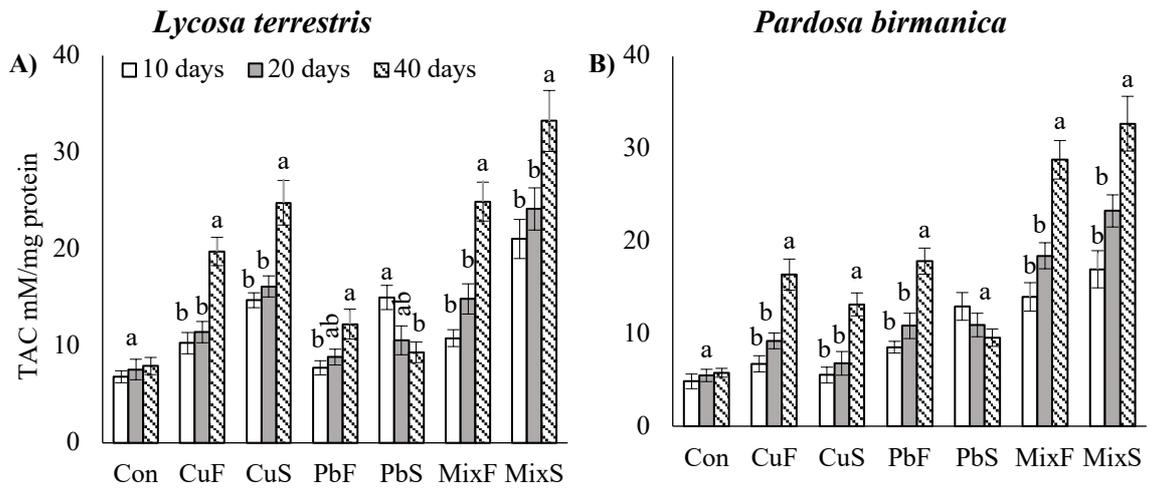
Exposure Routes	<i>Lycosa terrestris</i>						<i>Pardosa birmanica</i>				
	TAC	SOD	CAT	GPX	GR	TAC	SOD	CAT	GPX	GR	
Food	Cu	0.666*	0.879*	0.578*	0.719*	0.578*	0.685*	0.914*	0.862*	0.846*	0.862*
	TAC	1	0.691*	0.813*	0.654*	0.680*	1	0.655*	0.716*	0.718*	0.665*
	SOD		1	0.647*	0.860*	0.925*		1	0.875*	0.797*	0.852*
	CAT			1	0.639*	0.595*			1	0.878*	0.815*
	GPX				1	0.829*				1	0.831*
	GR					1					1
Soil	Cu	0.571*	0.856*	0.761*	0.809*	0.761*	0.547*	0.577*	0.592*	0.494*	0.592*
	TAC	1	0.595*	0.603*	0.639*	0.507*	1	0.394*	0.366*	0.441*	0.468*
	SOD		1	0.844*	0.827*	0.768*		1	0.467*	0.568*	0.592
	CAT			1	0.668*	0.679*			1	0.588*	0.574*
	GPX				1	0.661*				1	0.679*
	GR					1					1
Food	Pb	0.398*	0.830*	0.809*	0.776*	0.809*	0.786*	0.854*	0.868*	0.856*	0.868*
	TAC	1	0.533*	0.489*	0.389*	0.502*	1	0.717*	0.703*	0.678*	0.725*
	SOD		1	0.926*	0.813*	0.881*		1	0.890*	0.896*	0.891*
	CAT			1	0.826*	0.870*			1	0.832*	0.934*
	GPX				1	0.734*				1	0.851*
	GR					1					1
Soil	Pb	-0.367	-0.876*	-0.698*	-0.489*	-0.698*	-0.371	-0.723*	-0.739*	-0.039	-0.739*
	TAC	1	0.479*	0.409*	0.890*	0.407*	1	0.805*	0.709*	0.920*	0.484*
	SOD		1	0.714*	0.500*	0.686*		1	0.748*	0.687*	0.524*
	CAT			1	0.502*	0.645*			1	0.586*	0.686*
	GPX				1	0.829*				1	0.636*
	GR					1					1
Food	Cu + Pb	0.607*	0.915*	0.908*	0.787*	0.908*	0.785*	0.918*	0.852*	0.714*	0.852*
	TAC	1	0.777*	0.783*	0.723*	0.626*	1	0.805*	0.742*	0.613*	0.715*
	SOD		1	0.939*	0.878*	0.816*		1	0.837*	0.740*	0.889*
	CAT			1	0.867*	0.854*			1	0.667*	0.841*
	GPX				1	0.722*				1	0.730*
	GR					1					1
Soil	Cu + Pb	0.552*	0.874*	0.853*	0.831*	0.853*	0.603*	0.859*	0.818*	0.782*	0.818*
	TAC	1	0.564*	0.439*	0.523*	0.456*	1	0.619*	0.637*	0.645*	0.589*
	SOD		1	0.863*	0.853*	0.688*		1	0.907*	0.794*	0.847*
	CAT			1	0.752*	0.672*			1	0.794*	0.820*
	GPX				1	0.801*				1	0.769*
	GR					1					1

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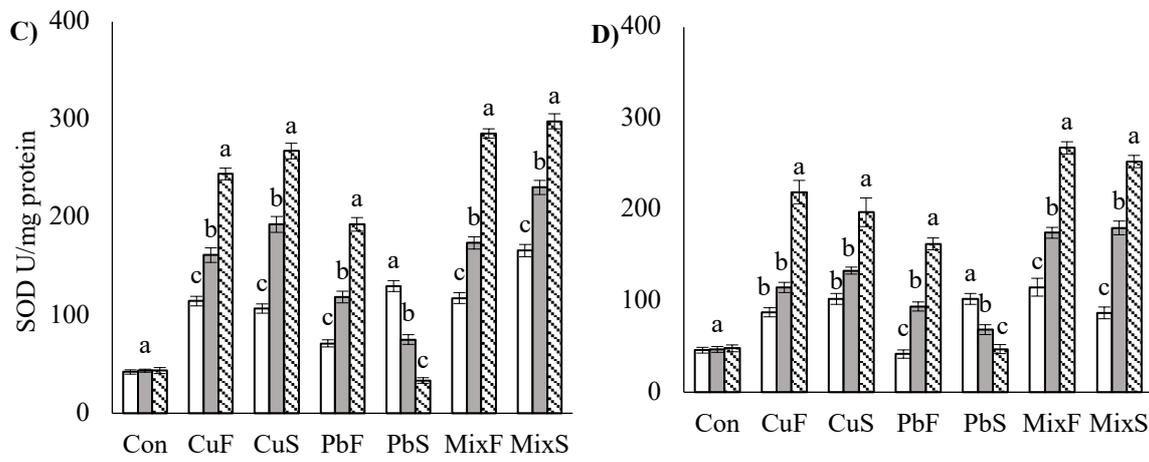
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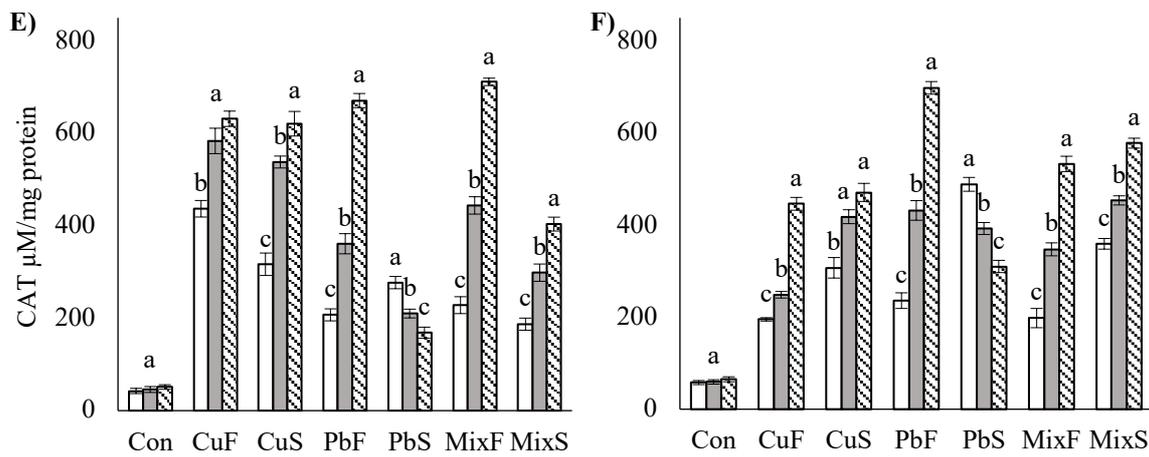
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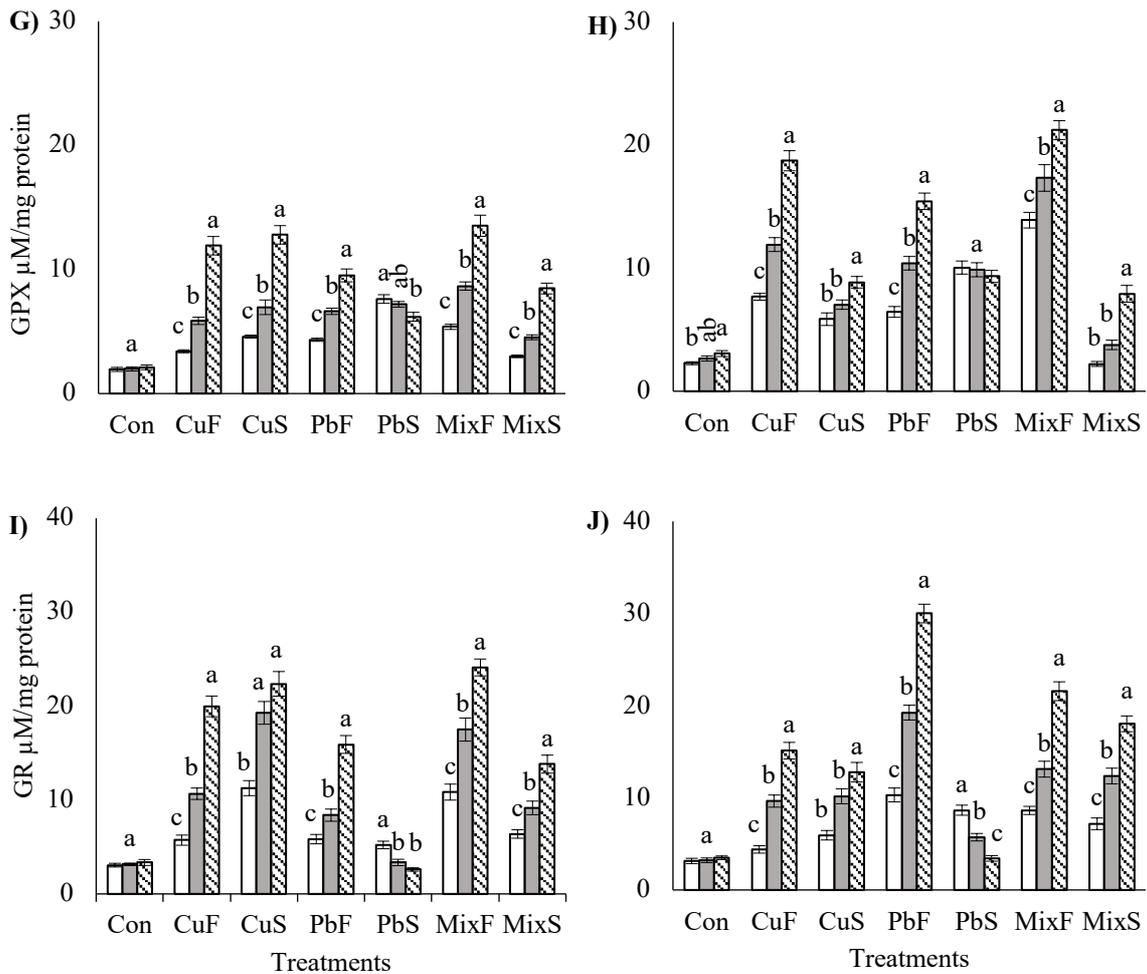


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824 Fig. 1 Total antioxidant capacity TAC (A, B), Superoxide dismutase SOD (C, D), Catalase CAT (E, F),  
 825 Glutathione peroxidase GPX (G, H) and Glutathione reductase GR (I, J) activities in *Lycosa terrestris* and *Pardosa*  
 826 *birmanica* after exposure to lead (Pb), copper (Cu) separately and in combination for 10, 20 and 40 days via  
 827 artificial food or soil. Treatments comprise Con (control), CuF (copper in food), CuS (copper in soil), PbF (lead  
 828 in food), PbS (lead in soil), MixF (lead and copper mixture in food) and MixS (lead and copper mixture in food).  
 829 Enzyme activities are expressed as mean  $\pm$  standard error (n = 10). Different superscripts letters show significant  
 830 difference between different exposure durations at  $P < 0.05$ .

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