SUPPLEMENTARY DATA

1 Materials and methods

1.1 Post-mitochondrial supernatant

The protocol to obtain the post-mitochondrial supernatant (PMS) that will be used for lipid peroxidation, glutathione *S*-transferases, glutathione peroxidase and catalase analysis was followed as described by Ferreira *et al.* (2010). Each replicate (two organisms) was homogenized using a sonicator (*Kika Labortechnik*, V200Scontrol, Germany) in 1ml K-Phosphate 0.1M buffer, pH 7.4. From the homogenate, 150µL were separated to a microtube and 5µL butylated hydroxytoluene (BHT) 4% in methanol were added for endogenous lipid peroxidation (LPO) determination. The remaining tissue homogenate (850 µL) was centrifuged at 10000g for 20 min. (4ºC) to isolate the Post-Mitochondrial Supernatant (PMS). The PMS was divided into four microtubes for posterior analysis of biomarkers and protein quantification. All microtubes were stored at -80ºC until analysis, for a period no longer than 2 weeks.

1.2 Lipid peroxidation

The lipid peroxidation (LPO) assay was based on the methods described by Bird and Draper (1984) and Ohkawa *et al.* (1979) and adapted to microplate by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm. The reaction included a mixture of 150 µL homogenated tissue, 500 µL trichloroacetic acid sodium salt (TCA) 12% (w/v), 500 µL 2-thiobarbituric acid (TBA) 0.73% (w/v) and 400µL Tris-HCl 60mM with diethylenetriaminepentaacetic acid **(**DTPA) 0.1mM. The reaction was carried out at 100ºC in a water bath for 1h. After this, samples were centrifuged for 5 min. at 11500 rpm (25ºC). Samples were kept away from light, at 25ºC and immediately read at 535 nm. LPO was expressed as nmol TBARS hydrolyzed per minute per mg of wet weight, using a molar extinction coefficient of 1.56 x10 M^{-1} cm^{-1}.

1.3 Glutathione *S*-Transferases

Glutathione *S*-Transferases (GST) activity was determined based on the method described by Habig *et al.* (1974). The PMS (100µL) was added to 200µL of a reaction solution and the result/substrate produced was measured at 340 nm. The reaction solution was a mixture of 4.95 ml K-phosphate buffer 0.1M (pH 6.5) with 900µL L-glutathione reduced (GSH) 10mM, and 150µL 1-chloro-2,4-dinitrobenzene (CDNB) 10mM. The enzymatic activity was expressed as unit (U) per mg of protein. A U corresponds to a nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 9.6 $x10^{-3}$ M^{-1} cm^{-1}.

1.4 Glutathione Peroxidase

Glutathione Peroxidase (GPx) activity was determined based on the method described by Mohandas *et al.* (1984). PMS (50µl) was mixed with 840µl K-phosphate buffer 0.05 M (pH 7.0), in a EDTA 1 mM solution, sodium azide 1mM and glutathione redutase (GR) (7.5mL from stock with 1 U/mL). Then 50 µL glutathione reduced (GSH) 4mM, NADPH and H_2O_2 (10 µl, 0.5mM) was added as substrate to the solution. The decrease in NADPH content (50µl, 0.8mM) was measured at 340 nm and the enzymatic activity expressed as unit (U) per mg of protein. A U corresponds to one nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 6.22$x10^{-3}$ M^{-1} cm^{-1}.

1.5 Catalase

Catalase (CAT) activity was determined based on the method described by Clairborne (1985). PMS (50µl) was mixed with 500µL H_2O_2 0.030M, and 950µL K-Phosphate 0.05M (pH 7.0) and the decomposition of the substrate (H_2O_2) measured at 240 nm. The enzymatic activity was expressed as unit (U) per mg of protein where a U corresponds to one µmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

1.6 Lactate dehydrogenase- sample preparation and reaction

Lactate dehydrogenase (LDH) activity was determined at 340 nm by the method of Vassault (1983) adapted to microplate by Diamantino *et al.* (2001). One animal (excluding the head) was homogenized using a sonicator in 500 µl of Tris/NaCl buffer (0.1M, pH 7.2), and the supernatants obtained after centrifugation (4 ºC, 4200g, 3 min) were removed and stored at -80ºC until enzymatic analysis. Activity determinations were made using 40µL of sample and 250µL of NADH (0.24mM) and 40µL of piruvate (10mM). The enzymatic activity was expressed as unit (U) per mg of protein. A U corresponds to one µmol of substrate hydrolyzed per minute, using a molar extinction coefficient of $6.3x10^{-3}$ M^{-1} cm^{-1}.

1.7 Acetylcholinesterase- sample preparation and reaction

One isopod head per sample was homogenized using a sonicator in 500µl of potassium phosphate buffer (0.1M, pH 7.2), and the supernatants obtained after centrifugation (4ºC, 3800g, 3 min) were removed and stored at -80ºC until enzymatic analysis. The AChE activity determination was performed according to the Ellman method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996).

In a 96 well microplate 250 µl of the reaction solution was added to 50µl of the sample and the absorbance was read at 414 nm, after 10, 15 and 20min. The reaction solution had 1ml of 5,50-dithiobis-2-nitrobenzoic acid (DTNB) 10mM solution, 1.280ml of 0.075M acetylthiocholine iodide solution and 28.920 ml of 0.1M phosphate buffer. The enzymatic activity was expressed as unit (U) per mg of protein. A U corresponds to one nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of $1.36x10^{-3}$ M^{-1} cm^{-1}.

1.8 Protein quantification for biomarkers

For all biomarker, protein concentration was determined according to the Bradford method (Bradford, 1976), adapted from BioRad's Bradford micro-assay set up in a 96 well flat bottom plate, using bovine γ-globuline as standard.

1.9 Energy reserves:

To determine total protein, carbohydrate and lipid contents, one isopod was homogenized using a sonicator in 1000µl distilled water after which was divided into three microtubes each one containing 300µl of the homogenate. The first fraction was used to determine protein and carbohydrate content, the second fraction to determine lipid content and the third fraction to determine the electron transport activity (ETS).

To determine total protein and carbohydrate contents, the homogenate was mixed with 100µl of 15% trichloroacetic acid (TCA) and incubated at -20ºC for 10 min (adapted from de Coen and Janssen (1997). After centrifugation (1000g, 10 min, 4ºC), the supernatant was separated as well as the carbohydrate fraction. The remaining pellet was resuspended in 1250µl sodium hydroxide (NaOH), incubated at 60 ºC for 30 min, after which it was neutralised with 750µl hydrocloric acid (HCl) and used as the protein fraction. Total protein content was then determined using the Bradford's reagent (Bradford 1976), and by measuring the absorbance at 590 nm using bovine serum albumin as a standard. Total carbohydrate content was determined by adding 50µl of 5% phenol and 200µl sulphuric acid (H_2SO_4) to 50µl of sample in a multiwell microplate, incubated for 30min at 20 ºC; the absorbance was measured at 492 nm using glucose as a standard. The protein and carbohydrate content is expressed as mg/mg organism (org) and J/mg org (expressed as fresh weight).

Total lipid quantification was based in the method described by Bligh and Dyer (1959). To the homogenate obtained was added 500µl chloroform (spectrofotometric grade). After vortexed, 500µl methanol (spectrofotometric grade) and 250µl bi-distilled water were added, centrifuged (1000g, 5min, 4ºC) and the top phase removed; the remaining phase was used for lipid measurement. 100µl of lipid extract plus 500µl H_2SO_4 were heated for 15 min (200ºC); after cooling down, 1.5ml of bi-distilled water was added and the total lipid content determined by measuring the absorbance at 375 nm using tripalmitin as a standard. The lipid content was expressed as mg/ mg org and J/mg org (expressed as fresh weight).

The electron transport activity was measured following de Coen and Janssen (1997) with the following modifications. To the homogenate obtained was added 150 µL of a buffer (0.3 M Tris-HCl pH 8.5, 45% (w/v) Poly Vinyl Pyrrolidone, 459 µM $MgSO_4$ and 0.6% (w/v) Triton X-100). Samples were then centrifuged at 1300 g during 10 min (4ºC).

The electron transport activity was determined by adding 50 µL of sample to 150 µL buffered substrate solution (0.13M Tris HCl, 0.3% (w/v) Triton X-100, pH 8.5, 1.7 mM NADH and 250 µM NADPH). The reaction was started by adding 100 µL INT (p-IodoNitroTetrazolium; 8 mM) and the absorbance measured at 490nm for 3 min.
The amount of formazan formed was calculated using a molar extinction coefficient of $15900 M^{-1} cm^{-1}$.

1.10 Cellular Energy Allocation (CEA)

The different energy reserve fractions (Ea): protein, carbohydrate and lipids obtained for the individual organisms were transformed into energetic equivalents using the energy of combustion described by Gnaiger (1983): 17.5 J/mg carbohydrate, 24 J/mg protein and 39.5 J/mg lipid. The cellular respiration rate (Ec) was determined using the ETS data, based on the theoretical stoichiometric relationship that for each 2 µmol of formazan formed, 1 µmol of O2 was consumed in the ETS system. The oxygen consumed per isopod was transformed into energetic equivalents using the specific oxyenthalpic equivalents for an average lipid, protein and carbohydrate mixture of 484 kJ/mol O2 (Gnaiger, 1983). The Ea, Ec and CEA value were calculated as described by Verslycke *et al.* (2004):

Ea (available energy) = carbohydrates + lipids + proteins (mJ / mg org.)
Ec (energy consumption) = ETS activity (mJ / mg org. / h)
CEA (cellular energy allocation) = Ea/Ec

1.11 Integrated Biomarker Response (IBR)

To try to integrate all results from different biomarkers and understand global/general responses, the integrated biomarker response (IBR) was calculated according to Beliaeff and Burgeot (2002). The IBR is calculated by summing up triangular Star Plot areas calculated for each two neighbouring data (biomarkers and energy reserves, time or temperature).

To calculate the IBR for biomarkers and energy reserves, the general mean (m) and the standard deviation (s) of all data regarding a given biomarker was calculated, followed by a standardization for each situation to obtain Y, where $Y = (X - m)/s$, and X is the mean value for the biomarker at a given concentration. Then Z was calculated using $Z = -Y$ or $Z = Y$, in the case of a biological effect corresponds respectively to an inhibition or a stimulation. Regarding the biological effect considered for each parameter, AChE and the energy related parameters: lipids, carbohydrates, proteins, energy available (Ea) and CEA were all assumed to decrease upon nickel exposure. In a similar way LPO rate was always assumed to increase with the exposure to the metal. The energy consumption (Ec) and LDH can either increase or decrease depending on the intensity of the stressor, and with organisms' strategy as well. In theory, organisms tend to spend more energy in order to deal with stressor, but an opposite strategy can also be used. In this case, organisms tend to decrease the energy that is directed to other physiological processes and thus resulting in an overall lower consumption, even lower than in situations where they were not under stress. In a similar way, the activity of the biomarkers GST and CAT can also be induced in order to cope with the formation of lipid peroxides or inactivated by ROS-mediated denaturation (Lizawa et al. 1994). For these reasons, their kinetics must be followed through time to consider their biological effect.

The score (S) was calculated by $S = Z+|Min|$, where $S \geq 0$ and $|Min|$ is the absolute value for the minimum value for all calculated Y in a given biomarker at all measurements made.

Star plots were then used to display Score results (*S*) and to calculate the integrated biomarker response (IBR) as:

$$IBR = \sum_{i=1}^{n} A_i$$

$$A_i = \frac{S_i}{2} \sin\beta \, (S_i \cos\beta + S_{i+1} \sin\beta)$$

$$\beta = \tan^{-1}\left(\frac{S_{i+1} \sin\alpha}{S_i - S_{i+1} \cos\alpha}\right)$$

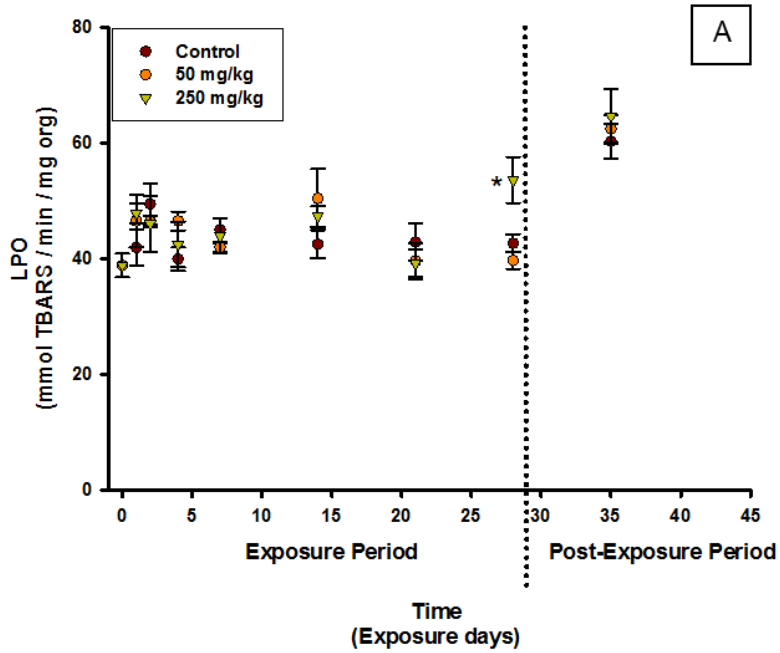
where S_i and S_{i+1} are two consecutive clockwise scores (radius coordinates) of a given star plot; A_i corresponds to the area the connecting two scores; *n* the number of biomarkers and energy reserves used for calculations; and $\alpha = 2\pi/n$.

In some sampling times, due to the high mortality obtained, it was not possible to determine all the parameters. Since the IBR is obtained by summing up all the parameters, to allow a correct and more accurate comparison it was divided by the number of sampling times and presented as IBR/n (Broeg and Lehtonen, 2006). Using this method it is possible to get an overall state of organisms for each parameter and each sampling time.

Analysing the scores as a fitness index, values that differed in 0.5 from the control score were considered to be from an animal with a higher or lower fitness (higher or lower scores, respectively).

The IBR calculations were always performed with the same order of parameters for all sampling times: the neurotoxicity biomarker AChE, followed by the detoxification and oxidative stress biomarkers GST, LPO and CAT, then the energy related biomarker LDH to serve as transition between biomarkers and energy related parameters and finally the lipids, carbohydrates and proteins content, the energy available (Ea), the energy consumption (Ec) and the CEA that integrates the last two parameters.

2 Figures



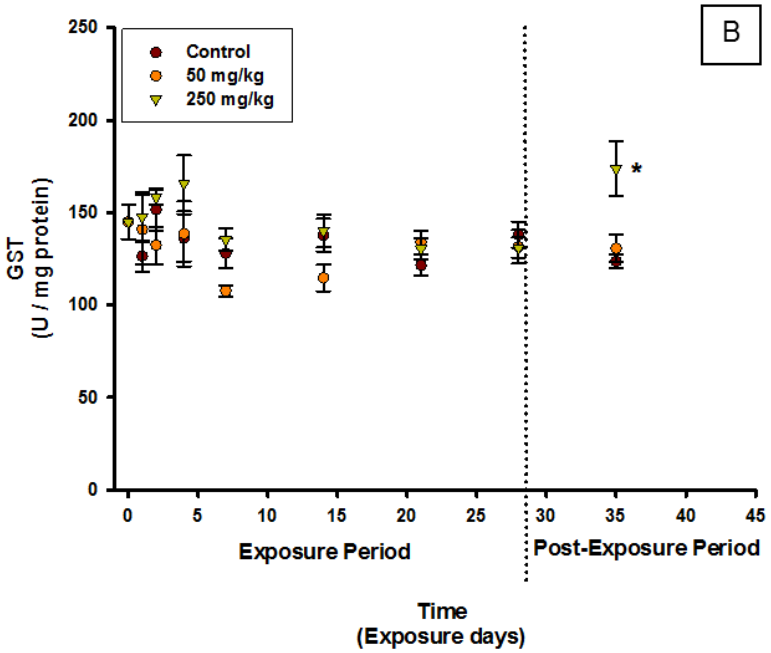
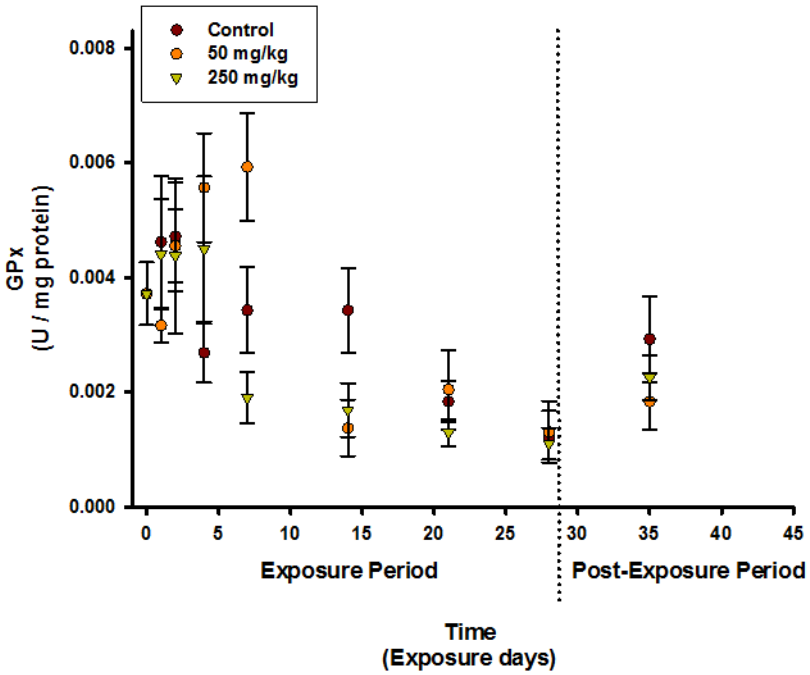
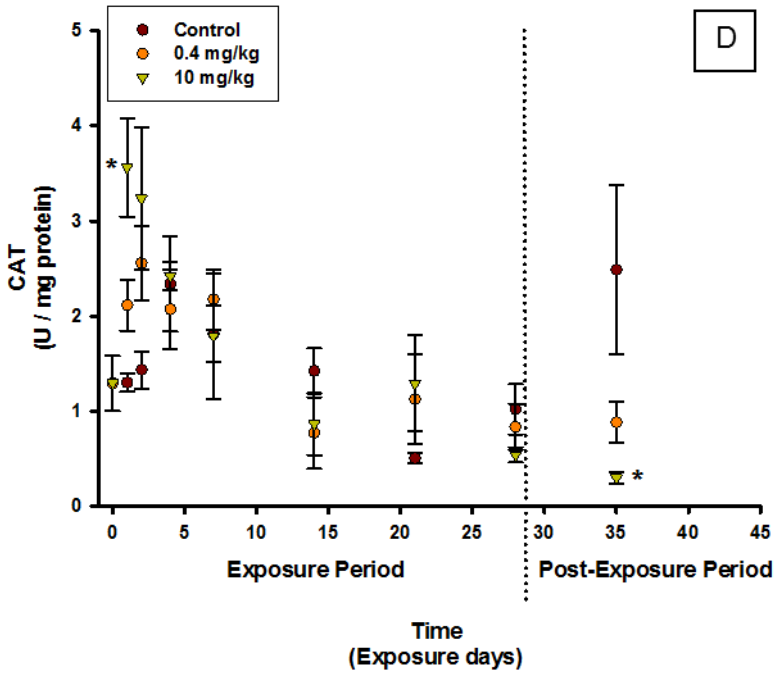


Fig. 1SD A-Lipid peroxidation rate (LPO), B- Glutathione *S*-Transferases, C- Catalase (CAT), D- Lactate Dehydrogenase (LDH), E- Acetylcholinesterase (AChE), F- Proteins , G- Carbohydrates, H- Lipids , I- Energy consumption, J- Energy available, K- Cellular Energy Allocation (CEA) in *Porcellionides pruinosus* exposed to nickel (Δ:50 mg / kg soil and □:250 mg / kg soil) for a 28 days period followed by a 14 days recovery in clean soil. Data represents mean values and corresponding SE. *= significant differences when compared with the control $p \leq 0.05$





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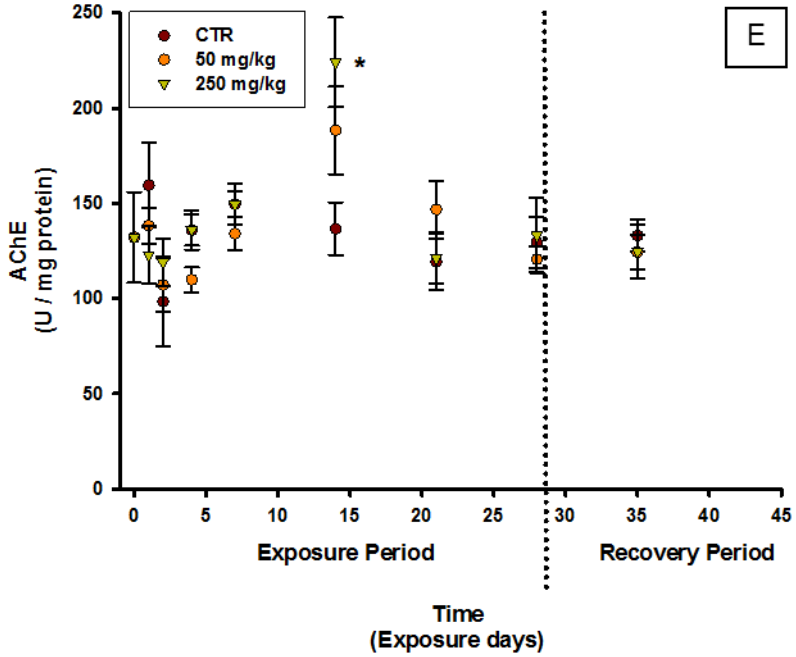

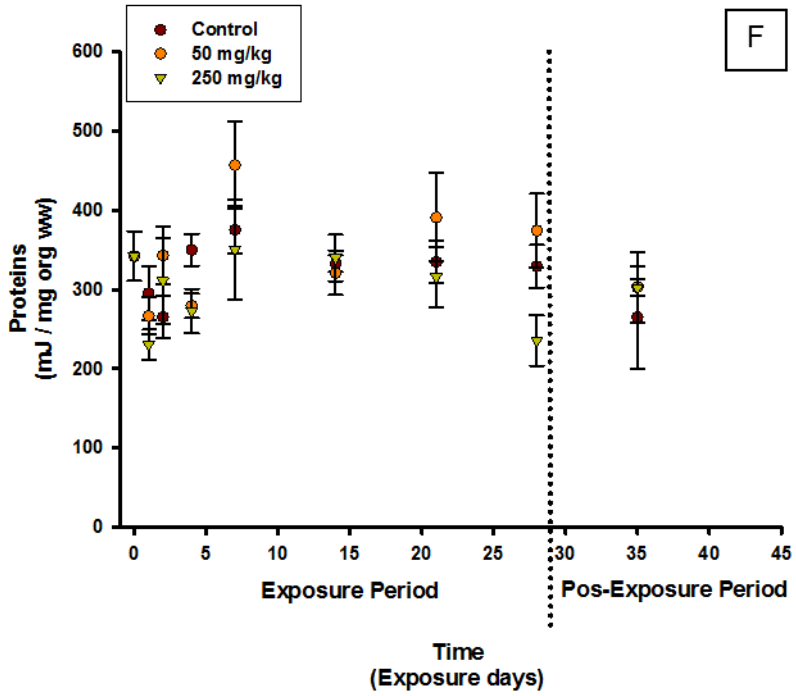

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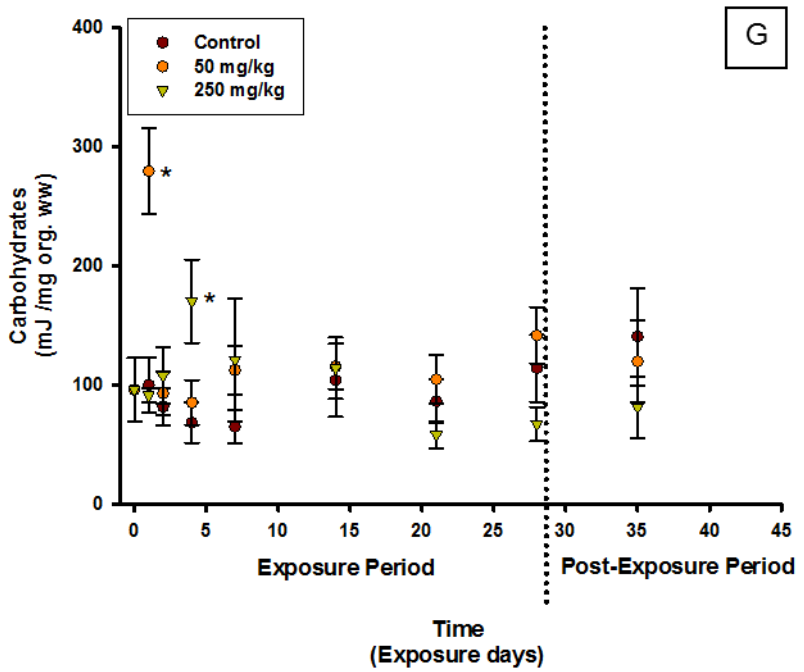

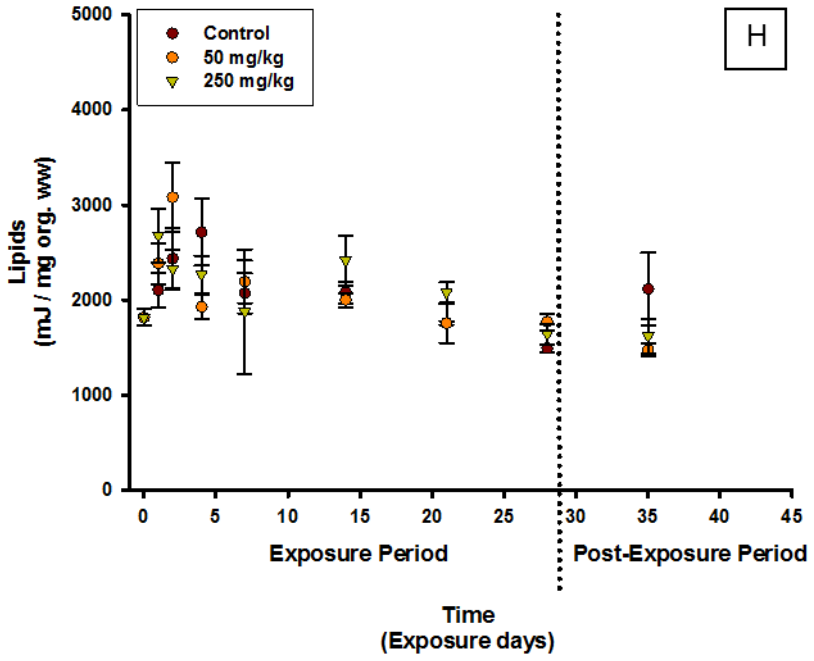

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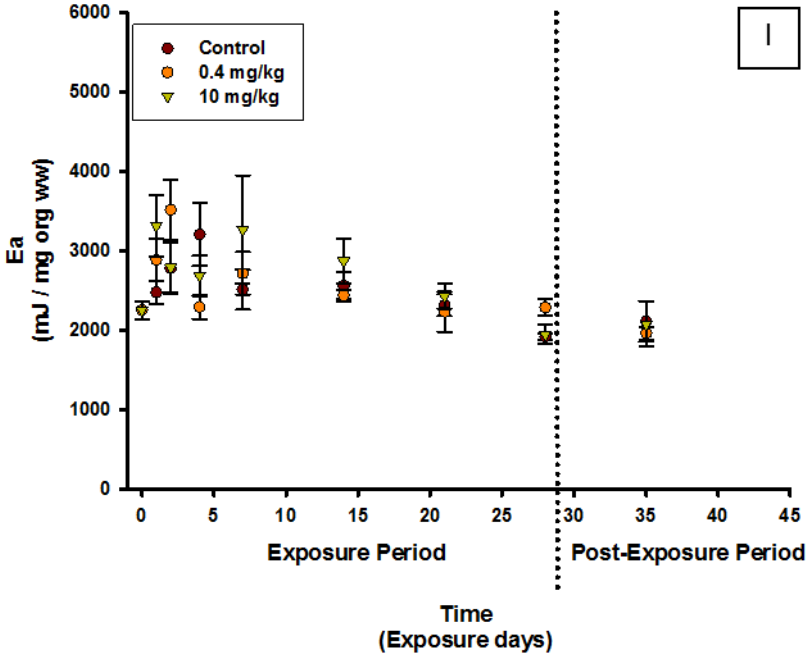

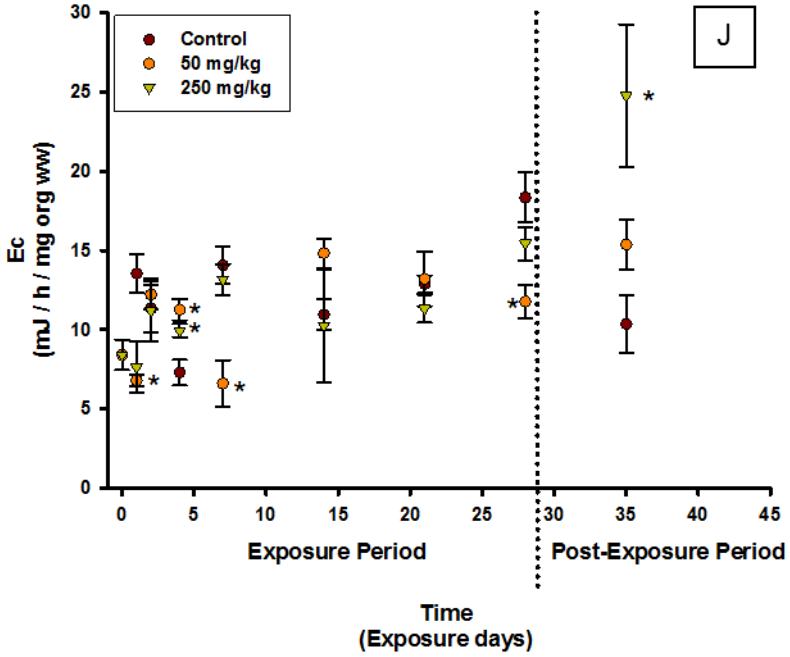

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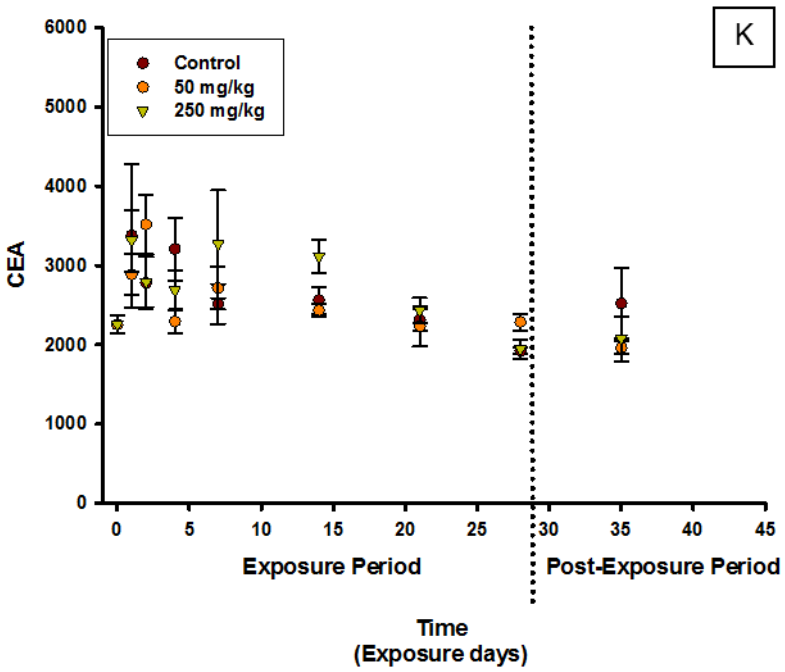

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3 Tables

Table 1SD IBR data comparison for biomarkers and energy reserves between control organisms from the species Porcellionides *pruinosus* and those exposed to nickel. Data refers to a 28 day exposure period followed by a 14 day recovery period. Red boxes denote deleterious effects, green boxes denote positive effects. ↗ - denotes increase, ↘ - denotes decrease; IBR is ± 0.5 from control's IBR value.

			AChE	GST	LPO	CAT	GPx	Lipids	Carbohydrates	Proteins	Ea	Ec	CEA
Exposure	24h	50 mg/kg			↘				↗			↗	↘
		250 mg/kg	↘	↘	↘	↗		↗		↘	↗	↗	
	48h	50 mg/kg		↗		↗		↗		↗	↗		↗
		250 mg/kg		↗		↗							
	96h	50 mg/kg	↘		↘		↘	↘		↘	↘	↘	↘
		250 mg/kg		↘			↘	↘	↗	↘			↘
	7d	50 mg/kg		↗			↘		↗	↗		↗	
		250 mg/kg					↗		↗		↗		↗
	14d	50 mg/kg	↗	↗	↘		↘						
		250 mg/kg	↗		↘		↘						↗
	21d	50 mg/kg								↗			
		250 mg/kg											
	28d	50 mg/kg										↗	
		250 mg/kg			↘				↘	↘			
Post-Exposure	35d	50 mg/kg				↗		↘				↘	↘
		250 mg/kg		↘	↘	↗		↘	↘			↘	