**TIME COURSE OF LEAD INDUCED PROTEOMIC CHANGES IN GILL OF THE
ANTARCTIC LIMPET *NACELLA CONCINNA* (GASTROPODA: PATELLIDAE)**

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ABSTRACT

The effect of increasing levels of metals from anthropogenic sources on Antarctic invertebrates is poorly understood. Here we exposed limpets (*Nacella concinna*) to 0, 0.12 and 0.25 $\mu\text{g l}^{-1}$ lead for 12, 24, 48 and 168 h. We subsequently quantified the changes in protein abundance from gill, using 2D gel electrophoresis and mass spectrometry. We identified several antioxidant proteins, including the metal binding *Mn*-superoxide dismutase and ferritin, increasing abundances early on. Chaperones involved in the redox-dependent maturation of proteins in the endoplasmic reticulum (ER) showed higher abundance with lead at 48 h. Lead also increased the abundance of *Zn*-binding carbonic anhydrase at 12 h, suggesting a challenge to acid-base balance. Metabolic proteins increased abundance at 168 h, suggesting a greater ATP demand during prolonged exposure. Changes in abundance of the small G-protein cdc42, a signaling protein modifying cytoskeleton, increased early and subsequently reversed during prolonged exposure, possibly leading to the modification of thick filament structure and function. We hypothesize that the replacement of metals initially affected antioxidant proteins and increased the production of reactive oxygen species. This disrupted the redox-sensitive maturation of proteins in the ER and caused increased ATP demand later on, accompanied by changes in cytoskeleton.

Key words: Acid-base balance, Antarctica, Cytoskeleton, Lead, Limpet, Oxidative stress, Protein homeostasis

SIGNIFICANCE

Proteomic analysis of gill tissue in Antarctic limpets exposed to different concentrations of lead (Pb) over a 168 h time period showed that proteomic changes vary with time. These changes included an increase in the demand of scavenging reactive oxygen species, acid-base balance and a challenge to protein homeostasis in the endoplasmic reticulum early on and subsequently an increase in energy metabolism, cellular signaling, and cytoskeletal modifications. Based on this time course, we hypothesize that the main mode of action of lead is a replacement of metal-cofactors of key enzymes involved in the scavenging of reactive oxygen species and the regulation of acid-base balance.

INTRODUCTION

The Antarctic environment is currently undergoing wide-ranging changes due to anthropogenic climate change and pollution [1]. One particular challenge is the transfer of metals from ice to the sea through increased melting and human pollution, affecting a number of Antarctic marine invertebrates, and in particular the limpet *Nacella concinna* [2].

Because of its toxicity to aquatic organisms and their potential to bioaccumulate, the effect of metals has been studied for several Antarctic marine species [3-8]. The relatively high concentrations of the metals Al, Co, Ni, Cu, Zn, Cd, Pb, Mn, Fe, As and Se in Antarctic waters have been attributed to water input from progressive deglaciation, soil composition, weathering of volcanic lithogenic rock and atmospheric deposition [9-13]. In addition, the natural background levels of trace metals have been elevated since the establishment of bases on the Antarctic continent [14-17]. During the austral summer the circumpolar vortex disappears and long-term records of mineral dust, black carbon and Pb in the South Pole and several coastal Antarctic stations indicate a greater transport of air masses poleward [18]. As a consequence, contamination of Antarctic snow by aerosols containing Pb has been widely documented and generally has been attributed to leaded gasoline [19], mining of non-ferrous metals, smelting or other anthropogenic sources in South America, Africa and Australia. Moreover, while the deposition of Pb in Antarctic snow has been decreasing in recent decades [20], metal emissions, including Pb, from human activities in Antarctica have the potential to cause environmental contamination in localized areas. For example, the main anthropogenic emissions within Antarctica are related to the burning of fuel for the operation of power plants, motorcycles, trucks, ships and aircrafts [21]. Indeed, the increased levels of Pb concentrations recorded in the proximity of the research stations indicated an anthropogenic origin. [13]. For example, a recent metal analysis of tissues from the Antarctic clam (*Laternula elliptica*) showed high Pb levels nearby an Antarctic research station [17]. In previous studies, Antarctica seawater showed elevated concentrations of lead in values between 0.05 to 0.06 μgL^{-1} , including places near the meltwater input source [10, 22, 23].

Evolutionary adaptations to the Antarctic environmental conditions can influence the sensitivity of organisms to stress [24-27]. Most biological processes in the

benthos and tidal zone of the polar seas, including growth, reproduction and metabolism are slow, which reduces the ability to detoxify and remove contaminants, making Antarctic organisms especially sensitive to additional environmental stress [28, 29]. In combination with the projections of increasing basal melt rates in the Weddell Sea sector of the West Antarctic ice sheet [30], the sensitivity to pollutants has the potential to be particularly stressful in the near future. Specifically, dissolved metals can be absorbed from the water column directly into the gills of mollusks [2]. Furthermore, the bioaccumulation of contaminants, resulting in the increase in tissue concentration, can cause harmful effects in aquatic invertebrates, even when concentrations in water are too low to carry the danger of acute toxicity [31].

Antarctic limpets have been widely used to study physiological responses to environmental stress [32-39]. Previous studies conducted in Antarctica, including King George Island, showed that *N. concinna* accumulated a considerable amount of metals in different tissues [9, 10, 40, 41]. Importantly, environmental stress, including exposure to different metals, led to the activation of the antioxidant system, an important indicator for the perturbation of the general redox system of the cell [2].

Proteomic analyses of the response to metals and other pollutants have been particularly successful in identifying novel patterns of cellular responses in temperate organisms [42, 43], characterizing unique changes in protein abundance in response to specific stressors [43-48], including limpets [49, 50]. Overall, the application of proteomics to study the response of Antarctic organisms to metal exposure can potentially provide insights into the effects of pollutants on the cellular responses in organisms with a slow metabolism and lower detoxification rate [51]. This is a promising approach, especially since proteomic studies on Antarctic organisms are largely absent [52, 53]. Our results from exposure of Antarctic limpets to Pb detected changes in several cellular processes associated with scavenging of reactive oxygen species, acid-base balance, protein homeostasis, energy metabolism, cellular signaling, and cytoskeletal modification, which offer insights into why cold-adapted organisms may be particularly sensitive to pollutant stress.

MATERIALS AND METHODS

Animal collection and maintenance

The Antarctic limpets (> 30 mm) were collected manually on rock surfaces at low tide during the austral summer 2010/2011 from King George Island, South Shetland Islands, Admiralty Bay at Plaza Point (62°04'14, 5"S; 58°24'11,9"W) and Botany Point (62°06'15,7"S; 58°21'14,0"W). Limpets were acclimated to laboratory conditions at the Brazilian Antarctic Station Comandante Ferraz for 72 h in an air-conditioned laboratory. Limpets were placed in recirculating aquaria. Salinity was 35 psu, water temperature 0°C and photoperiod 12/12 h. Genders were not distinguished. No mortalities occurred during the acclimation period.

Experimental design

Groups of limpets were exposed to lead dissolved in water at concentrations of 0.0 (control), 0.12 and 0.25 µg L⁻¹. The concentrations are, approximately, 2.5 and 5 times higher than those already observed in this particular environment. The exposure times were 12, 24, 48 and 168 h (N=6 for each time point and treatment). Lead was added to the aquaria at the start of the experiments from a stock solution. We also tested the control group for lead concentration. Gill tissue was sampled from all treatment groups and subsequently kept at -80°C. We choose to investigate gill tissue, as it is directly exposed to contaminated seawater, a metabolically highly active tissue and provides direct comparisons with other studies on the effects of metals on cellular function.

Homogenization

Frozen gill tissue was homogenized with an ice-cold ground-glass homogenizer in a ratio of 1:4 of tissue to homogenization buffer: 7 M urea, 2 M thiourea, 1% ASB-14 (amidosulfobetaine-14), 40 mM Tris-base, 0.5% immobilized pH 4–7 gradient (IPG) buffer (GE Healthcare, Piscataway, NJ, USA) and 40 mM dithiothreitol. The homogenate was subsequently centrifuged at room temperature for 30 min at 16,100 g and the supernatant was used for further processing. Proteins of the supernatant were precipitated by adding four volumes of ice-cold 10% trichloroacetic acid in acetone and incubating the solution at -20°C overnight. After centrifugation at 4°C for 15 min at 18,000 g, the supernatant was discarded and the remaining pellet was washed with ice-cold acetone, and centrifuged again before being re-suspended through vortexing in

rehydration buffer: 7 M urea, 2 M thiourea, 2% CHAPS (cholamidopropyl-dimethylammonio-propanesulfonic acid), 2% NP-40 (nonyl phenoxy)polyethoxylethanol-40), 0.002% Bromophenol Blue, 0.5% IPG buffer and 100 mM dithioerythritol. The protein concentration was determined with the 2D Quant kit (GE Healthcare), according to the manufacturer's instructions.

Two-dimensional gel electrophoresis

Proteins (400 mg) were loaded onto IPG strips (pH 4–7, 11 cm; GE Healthcare) for separation according to their isoelectric point (pI). We started the isoelectric focusing protocol with a 5 h passive rehydration step (0 V), followed by 12 h of active rehydration (50 V), using an isoelectric focusing cell (BioRad, Hercules, CA, USA). The following protocol was used for the remainder of the run (all voltage changes occurred in rapid mode): 500 V for 1 h, 1000 V for 1 h, and 8000 V for 2.5 h. The strips were frozen at –80°C. Frozen strips were thawed and incubated in equilibration buffer (375 mM Tris-base, 6 M urea, 30% glycerol, 2% SDS [sodium dodecyl sulfate] and 0.002% Bromophenol Blue) for 15 min, first with 65 mM dithiothreitol and then, second with 135 mM iodoacetamide. IPG strips were placed on top of a 12% polyacrylamide gel with a 0.8% agarose solution containing Laemmli SDS electrophoresis (or running) buffer (25 mM Tris- base, 192 mM glycine and 0.1% SDS). Gels were run (Criterion Dodeca; BioRad) at 200 V for 55 min with a recirculating water bath set at 10°C. Gels were subsequently stained with colloidal Coomassie Blue (G-250) overnight and destained by washing repeatedly with Milli-Q (Millipore, Billerica, MA, USA) water for 48 h. The resulting gel images were scanned with a transparency scanner (model 1280; Epson, Long Beach, CA, USA).

Gel image analysis

Digitalized images of two-dimensional gels were analyzed using Delta2D (version 4.3; Decodon, Greifswald, Germany) [54]. We used the group warping strategy to connect gel images through match vectors. All images within each metal concentration and time of exposure treatment were fused into a composite image (proteome map), which represents mean volumes for each spot. Spot boundaries were detected within the proteome map and transferred back to all gel images using match vectors. After background subtraction, protein spot volumes were normalized against total spot volume of all proteins in a gel image.

Mass spectrometry

Spots that significantly changed in volume in response to treatments were excised from gels using a tissue puncher (Beecher Instruments, Prairie, WI, USA). Gel plugs were destained twice with 25 mM ammonium bicarbonate in 50% acetonitrile, dehydrated with 100% acetonitrile and digested with 11 ng L⁻¹ of trypsin (Promega, Madison, WI, USA) overnight at 37°C. Digested proteins were extracted using elution buffer (0.1% trifluoroacetic acid [TFA]/acetonitrile; 2:10 and concentrated using a SpeedVac (Thermo Fisher Scientific, Waltham, MA, USA). The elution buffer containing the digested protein was mixed with 5 µL of matrix solution (0.2 mg m L⁻¹ -hydroxycinnamic acid in acetonitrile) and spotted on an AnchorchipTM target plate (Bruker Daltonics Inc., Billerica, MA, USA). The spots were washed with 0.1% TFA and recrystallized using an acetone/ethanol/0.1% TFA (6:3:1) mixture.

Peptide mass fingerprints (PMFs) were obtained on a matrix- assisted laser desorption ionization tandem time-of-flight (MALDI-TOF-TOF) mass spectrometer (Ultraflex II; Bruker Daltonics Inc.). We chose a minimum of six peptides to conduct tandem MS in order to obtain information about the b- and y-ions of the peptide sequence. To analyze the peptide spectra we used flexAnalysis (version 3.0; Bruker Daltonics Inc.) and applied the TopHat algorithm for baseline subtraction, the Savitzky–Golay analysis for smoothing (with: 0.2 *m/z*; number of cycles) and the SNAP algorithm to detect peaks (signal-to-noise ratio: 6 for MS and 1.5 for MS/MS). The charge state of the peptides was assumed to be +1. We used porcine trypsin for internal mass calibration. To identify peptides we used Mascot (version 3.1; Matrix Science Inc., Boston, MA, USA) and combined PMFs and tandem mass spectra in a search against two databases. One database included the sequences of Patellogastropoda that contained 1512528 sequences and 346221826 residues (NCBI). The second database used was NCBI National Center for Biotechnology Information (Bethesda, MD, USA) with 4626804 sequences and 1596079197 residues (Mollusca). In the event of peptides matching to multiple members of a protein family, we selected the protein with the highest score and the highest number of matching peptides. Search parameters were set as follows: enzyme specificity: trypsin; one missed cleavage permitted; variable modifications: carbamidomethylation of cysteine and oxidation of methionine. For

tandem MS we set the precursor ion mass tolerance to 0.6 Da. For the molecular weight search (MOWSE) scores higher than 46 indicated a significant hit for both databases ($P < 0.05$). Nevertheless, we only accepted positive identifications that included two matched peptides regardless of the MOWSE score.

Statistical analysis

Normalized spot volumes were analyzed within Delta2D (version 4.3; Decodon, Greifswald, Germany). We conducted a two-way permutation ANOVA to estimate the relative importance of the factors metal concentration and exposure time. For the two-way ANOVA, a null distribution was generated using 1000 permutations to account for the unequal variance and non-normal distributions of the response variables, and a P -value of 0.02 was used instead of doing a multiple comparison correction, given the limited number of proteins. Following the two-way ANOVA, *post-hoc* testing to compare treatments was conducted using Tukey's analysis ($P < 0.05$), using Minitab (version 16; Minitab Inc., State College, Pa, USA). For the hierarchical clustering we used average linking within the statistical tool suite within Delta2D, using a Pearson's correlation metric.

In addition we used a principal component analysis (PCA) within Delta2D to assess the variation among treatments and to evaluate the contribution of single proteins to the variation. The results of the PCA comparing treatments were interpreted with the help of loadings; we used these plots to assess which proteins contributed the most to the component of interest.

RESULTS AND DISCUSSION

Proteomic analysis of gill tissue of *N. concinna* in response to various lead concentrations generated a fused image, or proteome map, comprising 72 2D gels (Fig. 1; $N=6$ for each time point and treatment). After careful reviewing and editing spot boundaries on this fused image, we detected 319 protein spots. Of these, 64.9% (207) changed abundance in response to exposure time, lead concentration (main effects) or their interaction (two-way permutation ANOVA; $P \leq 0.02$). We were able to identify 42.1% (87) of the proteins that changed (or 27.3% of the total), using tandem mass spectrometry (Table S1). In addition to indicating the effect of lead concentration (C),

exposure time (T) and their interaction (I) based on the entire data set within each heat map, we also conducted separate pairwise comparisons for each protein showing significant changes in abundance (Tukey post-hoc pairwise comparison based on a general linear model; $P \leq 0.05$), which we indicated in figures presenting changes in abundance for individual proteins.

It is important to consider that protein abundance can change because of synthesis, post-translational modifications (PTMs) or degradation. Thus, when we refer to abundance changes of protein spots, one or a combination of these processes may be responsible. Furthermore, it is possible that we did not detect all isoforms of a protein because they are outside the pH or mass range we analyzed and thus that the overall sum of the detected protein isoforms does not represent the total abundance of the protein. Thus, the inferences we put forward herein are considered hypotheses, which require further validation. We will first discuss the results of principal component analyses and then the cellular processes associated with the function of specific proteins organized by categories: oxidative stress, protein homeostasis, energy metabolism, and cytoskeletal proteins.

Principal component analyses (PCAs)

PCAs were conducted to assess the contribution of time and lead exposure, or their interaction, to the variation in protein abundance (Fig. S1-S3). Given the lack of a distinct separation when including all treatments and the difficulty in interpreting the results, we simplified the interpretation by conducting PCAs for each time point separately (Figs 2A-D). Based on the changes in abundance of 87 proteins, the control group separated from those exposed to lead at all four time points (12, 24, 48 and 168 h) along the x-axis (PC1), explaining 19-29% of the variation. The greatest variation of 29% after the longest exposure time (168 h or seven days) suggested that the effect of lead increased with time.

PC2 separated the $0.12 \mu\text{gL}^{-1}$ from the $0.25 \mu\text{gL}^{-1}$ treatments for each time point. Interestingly, at 12 h, a single outlier seemed to have contributed majorly to the separation (along PC2) of the two lead exposure treatments. With exception of this one outlier, both treatments are less separated at 12 h than at any other time point (Fig. 2A). These results show that the effects of elevated lead levels started to show as early as 12 h into the exposure, but increased further with time.

The loadings provide a first exploratory assessment of the possible changes that accompany the separation of treatment groups. A comparison of loadings from different functional protein categories along PC1, showed positive loadings of at least one of three antioxidant proteins (indicated in gray). This includes the iron-binding ferritin, the mitochondrial *Mn*-superoxide dismutase (*Mn*-SOD), and aldehyde dehydrogenase (ALDH7), which is involved in the detoxification of aldehydes originating from lipid peroxidation [55] during all four time points (Tables 1-4). The latter suggests that lead caused a change in the abundance of at least one of these three antioxidant stress proteins at all times.

Negative loadings, meaning lower abundances during lead treatment at 12, 24 and 48 h (Tables 1-4) were found for several metabolic proteins (indicated in blue). The tricarboxylic acid (TCA) cycle reactions of pyruvate dehydrogenase (PDH), malate dehydrogenase (MDH) and dihydrolipoyl dehydrogenase (DHLDH), which is a subunit of either the PDH or the α -ketoglutarate dehydrogenase enzyme complex, were among those proteins. Phosphorylase b kinase, which regulates glycogen phosphorylase and thus glycogen metabolism [56], showed also negative loadings (lower abundances with lead) at 48 h. Taken together, these results indicate a decreased level of proteins involved in specific metabolic pathways, like the TCA cycle, early on. The most abundant category are cytoskeletal proteins (yellow) mainly represented by thick and thin filaments, myosin heavy and regulatory light chains (MHC and MLC) as well as paramyosin and actin in PC1 at all four time points. However, MHC, MLC and paramyosin showed opposite (negative at 12 h and positive at 48 h) loadings for PC1, suggesting that their contribution to the separation changed over time (Tables 1-4).

Proteins belonging to the functional category of protein homeostasis, the endoplasmic reticulum calreticulin and protein disulfide isomerase and glucose-regulated protein 78 (GRP78), as well as the actin chaperone T-complex protein 1 β , showed positive loadings at 12, 24 and 168 h (red), possibly indicating a greater requirement to increase re-folding of denaturing proteins upon lead exposure.

Finally, several signaling proteins showed high loadings along PC1 (Tables 1-4). The small G-protein cdc42, a key regulator of cytoskeletal structures under stress [57], showed both, lower and higher abundances with lead exposure at 24 and 48 h, respectively.

PC2 separated the effect of the lower versus the higher lead concentrations. Positive loadings generally indicate higher abundances in the lower concentration

whereas negative loadings generally indicate higher protein abundances at the higher lead concentration. It is important to state that we see different responses to the two concentrations with time.

Proteins involved in energy metabolism, specifically the glycolytic enzymes phosphoglycerate kinase and α -enolase, and the TCA cycle NADP-dependent isocitrate dehydrogenase (NADP-ICDH), showed higher negative loadings for PC2 at 12 h. PDH, however, showed positive loadings at $0.12 \mu\text{gL}^{-1}$ lead at 12 h, suggesting a higher activity of glycolysis at the higher lead concentration (Table 1). This trend continues at 24 h, with two isoforms of triose phosphate isomerase (TPI) and arginine kinase (AK), which temporarily buffers the increased ATP demand during stress, showing higher negative loadings at $0.25 \mu\text{gL}^{-1}$ lead. Two PDH protein isoforms also contributed to PC2 at 48 h and 168 h. Other metabolic proteins, including arginine kinase, NADP-ICDH, DHLDH and methylmalonate-semialdehyde dehydrogenase (MMSDH) contributed to PC2 with generally negative loadings at $0.25 \mu\text{gL}^{-1}$ lead at 168 h. Overall, metabolic, mainly glycolytic, proteins contribute more to negative loadings to PC2, which generally accompanies higher abundances, and possibly greater glycolytic rates, at $0.25 \mu\text{gL}^{-1}$ lead over the 168 h time course.

One ferritin isoform contributed to the positive and two contributed negative loadings to PC2 at 48 and 168 h. Another antioxidant protein, peroxiredoxin1/2 (PRX1/2), contributed positive loadings at 168 h to PC2. Thus, the contribution of both ferritin and PRX1/2 to PC2 suggest that there response to lead is dose-dependent during prolonged exposure.

Again, the most abundant functional group, cytoskeletal proteins (mainly actin and MHC/MLC), were represented among the proteins contributing to PC2, especially at 12, 24 and 48 h, but less so at 168 h (Tables 1-4). In this context, it might be important that two different protein isoforms of cdc42 contributed high negative loading for PC2 at 12 and 24 h, possibly affecting the signaling cascade leading to cytoskeletal changes.

Finally, at 12 and 24 h, serine/threonine-protein phosphatase and cdc42 showed positive and negative loadings for PC2, possibly representing different and opposing signaling pathways affecting MLC. Both, phosphorylase b kinase, which is involved in glycogen metabolism, and calcium-dependent protein kinase, which is involved in the

phosphorylation of MLC isoforms, contributed to PC2 at 168 h (Table 2D). Again, this means that their response to lead is dose-dependent.

Oxidative stress

The abundances of the antioxidant proteins *Mn*-SOD, PRX1/2, ALDH7 and ferritin, changed throughout the entire exposure (Figs 3 and 4). In cluster I, both *Mn*-SOD and two protein isoforms of ferritin increased with at least one of the lead concentrations at 12, 24 and 168 h. In cluster II, PRX1/2 and ALDH7 showed a dose-dependent increase in abundance. In cluster III, two ferritin isoforms mainly showed an increase in abundance with time independent of lead exposure.

Mn-SOD converts superoxide anions (O_2^-) to hydrogen peroxide (H_2O_2), which is subsequently reduced to water by PRX1/2, requiring NADPH as the reducing equivalent to reduce oxidized PRX through the reactions of thioredoxin and thioredoxin-reductase [58, 59]. Although both reactions are linked, because *Mn*-SOD and PRX1/2 are localized in the mitochondrion and cytosol, respectively, they are not clustering together. It is possible that PRX1/2 generally functions as an early response protein to elevated levels of H_2O_2 , as it has been shown to signal the presence of higher levels of H_2O_2 by oxidizing other proteins [60].

The ferritin holoenzyme is a 24-mer that can contain different ratios of heavy (H) or light (L) subunits [61]. We were able to identify two of the isoforms (spots#188 and 66) as most likely representing the heavy chain, which has ferroxidase activity and thus is able to convert Fe^{2+} to Fe^{3+} , preempting the Fenton reaction [62]. While the first of these two (spot#188) showed higher abundance for at least one of the two lead treatments at 12 and 24 h, the second one (spot#66) showed overall higher levels with time (regardless of lead), starting at 24 h (Figs 3 and 4).

The other two isoforms of ferritin (spot#162, 316) could not be unequivocally identified as either one of the subunits. One of them (spot#162), clustering with *Mn*-SOD, showed higher abundance for at least one of the two lead treatments relative to the control throughout the entire exposure. The other one (spot#316) showed an overall increase in abundance with time regardless of lead. In contrast to the heavy chain, the light chain lacks the ferroxidase activity but instead facilitates the storage of iron and thereby sequesters Fe^{2+} from the cytosol, again, to prevent the Fenton reaction from

generating hydroxyl radicals [62]. Thus, either subunit can contribute to the antioxidant activity of the cell. Ferritins have been isolated in a number of marine mollusks [63; and references therein], including limpets [64, 65], and are likely involved in the innate immune response to pathogens by withholding iron [66]. Furthermore, their abundance is also regulated by other metals such as Fe, Cu and Cd [67-69]. Ferritin was induced after 6 h of exposure to Cd, Pb and Fe in the heteronemertean worm *Dendrorhynchus zhejiangensis* [70] and after four weeks of simultaneous exposure to both elevated P_{CO_2} and Cu in the oyster *Crassostrea virginica* [71]. In contrast, exposure to Cd, Cu and Zn lowered the abundance of the ferritin transcript in the oyster *Saccostrea glomerata* [72], suggesting that metals might have different effects on the transcriptional and translational regulation of ferritin.

The abundance changes of the two ferritin isoforms of cluster I early on represent the most indicative response to lead, most likely due to the cell responding to an increase in ROS, which can, if not scavenged, disrupt various cellular structures and functions, e.g. the cytoskeletal dynamics, in other mollusks [73-75].

In summary, antioxidant proteins, specifically ferritin and Mn-SOD were induced upon lead exposure. Both proteins are metal-binding proteins and it is possible that lead competes with the binding of these metals. If this affects the function of these proteins, it is possible that the generation of ROS will increase and affect other cell components. Protein damage by ROS can in turn challenge protein homeostasis.

Protein homeostasis

Proteins that responded to elevated levels of lead involved several molecular chaperones, proteases that eliminate aberrant proteins and proteins regulating protein synthesis [76]. Stress conditions can lead to the misfolding of proteins, which, if they cannot be refolded, will have to be degraded; otherwise they increase the rate of the formation of protein aggregates. In addition, *de novo* protein synthesis is prone to generate misfolded proteins as the proteins emerging from the ribosome are not fully folded and thus are more likely to expose hydrophobic side chains that are normally buried inside the protein. It is known that metals interfere with protein folding *in vivo*, presenting one of the main mechanisms of metal toxicity [77]. Metal toxicity derives, besides from impaired protein folding, from the competition with essential metal ions and the formation of metal protein complexes.

The proteins we identified include five molecular chaperones, three proteasome subunits and one translation initiation factor (Figs. 5 and 6). Cluster I showed changes in abundance of two chaperones: T-complex protein 1 (TCP1), an actin and tubulin chaperone [78], and the ER PDI both showed interaction effects and contributed positive loadings to PC1 at 168 h (Fig. 2D and Table 4). Both proteins showed significantly greater abundances with elevated lead at 48 h (Fig. 6), indicating that it took at least two days of exposure to disrupt protein homeostasis.

Cluster II included one isoform of the ER calreticulin and the eukaryotic translation initiation factor 3 (eIF3) (Fig. 5). Overall, abundances of both of these proteins decreased with elevated lead, although only the abundance of eIF3 at 12 h was significantly lower at $0.25 \mu\text{gL}^{-1}$ (Fig. 6). The lower abundance of eIF3 suggest a reduction in protein synthesis [79], and possibly lower levels of total protein, as has been shown in the green mussel *P. viridis* upon chronic lead exposure [80].

Cluster III included three proteasome subunits and the two ER chaperones calreticulin and glucose-regulated protein 78 (GRP78) (Fig. 6). The most common response among these proteins was increased levels at $0.12 \mu\text{gL}^{-1}$ lead at either 48 or 168 h. The dose-dependent response of these proteins to lead is further supported by their contributions, i.e. high loadings, of three of these proteins to PC2 at several time points (Fig. 2 and Tables 1-4).

The four ER chaperones [81, 82] PDI, GRP78 and the two isoforms of calreticulin, responded to lead in a time-dependent matter. These results suggest that lead exposure affects protein folding and maturation in the ER, e. g. glycosylation in case of calreticulin [83]. Furthermore, the ER compartment is more oxidized than other cellular compartments to facilitate the formation of disulfide bonds by PDI [84]. Any challenges to the redox balance of the cell therefore quickly affect protein maturation in the ER [85]. Finally, maturation of secretory proteins in the ER is closely linked to the transport of vesicles via the Golgi apparatus and tubulin providing the microtubular structures for vesicle transport. Thus, the observation that a tubulin chaperone is affected by lead exposure, in addition to several ER chaperones, further supports the hypothesis that lead caused a challenge to protein maturation in the ER.

The elevated levels of proteasome subunits suggested that low levels of lead ($0.12 \mu\text{gL}^{-1}$) induce greater levels of protein degradation than higher levels at 48 and 168 h. It is likely that degradation by removing aberrant proteins and preventing the

formation of protein aggregates, is a response towards reestablishing protein homeostasis and that the lack of this response at the higher lead concentration might present the underlying mechanism of lead toxicity [86].

In summary, exposure to lower levels of lead caused an increase of protein degradation (cluster III) while simultaneously inhibiting part of the cellular machinery in charge of maintaining protein homeostasis, e.g. TCP1, early (cluster I) and in the ER later during exposure. Lead ($0.25 \mu\text{g L}^{-1}$) may also interfere with the initiation of protein synthesis (eIF3) early on. Our results strongly support the hypothesis that lead affected protein homeostasis, inducing protein degradation and challenging protein maturation, in *N. concinna* gill.

Energy metabolism

Proteins representing phosphotransferases, glycolysis and the tricarboxylic acid (TCA) cycle were all identified to be responding to lead (Figs 7 and 8). In addition, several isoforms of carbonic anhydrase (CA) indicated that lead affected the formation of bicarbonate from metabolic CO_2 and therefore acid-base balance. We will also discuss the signaling protein phosphorylase kinase α (β subunit), as it is involved in the breakdown of glycogen via glycolysis [56, 87].

Cluster I included PDH E1 β and two isoforms of CA, which increased abundance with lead at 12 h. Mitochondrial malate dehydrogenase (mMDH) increased at $0.12 \mu\text{g l}^{-1}$ only (Fig. 8A). One CA isoform (spot#204) maintained higher abundance upon lead exposure until 48 h, which after that, however, reversed. Both CA isoforms contributed high loadings to PCs at 12, 48 and 168 h (Tables 1-4). The PDH isoform (spot#198) did so as well at 12 h. Thus, the proteins of this cluster greatly contributed to the separation of treatment groups and increased abundances early during the time course.

Cluster II included the E1 subunit of PDH, dihydrolipoyl dehydrogenase (DDL DH) and phosphorylase kinase β , two isoforms of triose phosphate isomerase (TPI) and methylmalonate semialdehyde dehydrogenase (MMSDH), which is part of the valine oxidation pathway [88, 89] (Fig. 7). The common feature of this cluster is a decrease of abundances over time at the higher lead concentration. Both phosphorylase kinase β and TPI showed significantly lower abundance at the later time points (24, 48

and 168 h) for one of the lead concentrations (Fig. 8B, C). The former also contributed to PC2 at 168 h (Table 4C).

Two out of four proteins showed lower abundance with lead at 12 h in cluster III: PDH E1 α (spot#110) and CA (spot#197) (Fig. 7C). Arginine kinase and the glycolytic phosphoglycerate kinase showed interaction effects, but no significant pairwise comparisons.

Cluster IV included eight proteins, three of which showed higher abundances with one of the lead concentrations relative to the control at 24 h, six did so at 168 h, but two of those also showed lower abundances with lead at 48 h (Figs. 7 and 8D). Among those proteins that showed greater abundance with lead at 168 h are isoforms of arginine kinase, the glycolytic TPI and enolase, the E1 subunit of PDH, DHLDH and O-glycosyl hydrolase. Thus, prolonged exposure to lead increased arginine kinase, two glycolytic proteins, PDH and a TCA cycle protein, suggesting an increase in metabolic demand. The increase in the abundance of these proteins during prolonged exposure contrasts with the protein changes in cluster I, which increased abundance early on.

One of the two arginine kinase isoforms (spot#274) was ten-fold induced at 168 h. Two TPI isoforms showed lower, one TPI (spot#224) revealed higher abundance with lead at 168 h. TPI levels could be shifting due to PTMs or to accelerate the rate of glycolysis. An increase of overall glycolysis is also supported by an increase of α enolase at $0.25 \mu\text{gL}^{-1}$ at 168 h. The third glycolytic protein, phosphoglycerate kinase, did not show any significant differences at 168 h. Of the four PDH isoforms, one was low-abundant (spot#132), two medium-abundant (spots#110 and 14) and one highly abundant (spot#198). One of the medium-abundant isoforms (spot#14) increased with lead at 24 h and 168 h, the highly abundant one did so at 12 h. The TCA cycle proteins NADP-ICDH, DHLDH (two protein species) and mMDH, showed no consistent changes. The two DHLDH isoforms showed lower (spot#244) and higher (spot#254) abundances with lead at 12 h and 168 h, respectively, possibly shifting PTMs. One MMSDH decreased abundance with $0.12 \mu\text{gL}^{-1}$ relative to the control at 168 h. MMSDH is involved in valine metabolism, which, together with the metabolism of other branched-chained amino acids, resembles in many ways the β -oxidation of fatty acids [90], and can respond in opposite direction to glycolysis in response to a high fat load in humans [89].

In conclusion, exposure to lead for 168 h increased the abundance of a number of metabolic proteins representing phosphotransferases, glycolytic reactions and the reaction of pyruvate dehydrogenase but not those of the TCA cycle, with DHLDH (spot#254) being the exception. Lead has been shown to affect enzymes involved in carbohydrate metabolism, including glycolysis, in rats and humans, in part through the induction of insulin resistance [91, 92].

Of the three carbonic anhydrase isoforms, two that differed by mass clustered together (cluster I) and showed higher abundance with lead at 12, 24 and 48 h (spot#204 only). The latter isoform reversed this patterns and showed lower abundance relative to the control at $0.25 \mu\text{gL}^{-1}$ at 168 h. The third isoform (spot#197) showed lower abundance with lead at 12 h. CA activity is important for acid-base balance and ion transport, specifically in the gill [93, 94]. Our results suggest that the effect on the abundance of CA was isoform-specific. If these changes in abundance are increasing activity or are an indication of compensating for an inactive enzyme and the overall effect is still one of lower CA activity, is unclear.

Phosphorylation of the β -subunit of phosphorylase kinase *a* converts the inactive phosphorylase *b* into the active *a* form, which catalyzes glycogenolysis [56, 95]. Importantly, phosphorylase kinase *a* showed lower abundance with lead, significantly so at 12, 24 and 48 h, suggesting that its activity of phosphorylating phosphorylase *b* and thus catalyzing the breakdown of glycogen decreased with lead. However, since the β subunit of phosphorylase kinase has to be phosphorylated to form the fully active holozyne [56], the effect of this isoform on glycogen metabolism depends on identifying its phosphorylation status, which we weren't able to do.

In summary, given these results we hypothesize that lead increased the abundance and possibly the activity of CA and thus affected acid-base balance at 12 h and that prolonged exposure (168 h) induced an increase in arginine kinase and glycolysis in gill of *N. concinna*.

Cytoskeletal proteins

We can distinguish five clusters of changing protein abundances related to the cytoskeleton (Fig. 9). Besides a number of actin and myosin heavy and light chain isoforms (MHC and MLC, respectively), we identified smooth muscle paramyosin,

tropomyosin, calponin and filamin C, which directly interact with actin filaments [96]. In addition, we included several signaling proteins that are involved in cytoskeletal rearrangements during stress (*cdc42*), affect MLC activity through changing phosphorylation (serine/threonine phosphatase 2A and Ca-dependent protein kinase 2) and vesicular transport involving the cytoskeleton (ADP-ribosylation factor).

The first cluster included five actin isoforms with consistently higher molecular mass (~60 kDa) than typical for the monomeric actin (~45 kDa). The abundance of these actins generally increased at 0.25 μgL^{-1} at 24 h, and 0.12 μgL^{-1} lead at 48 h, but at 168 h the abundance of all isoforms decreased with increasing lead (Fig. 9).

Cluster II included eight actins, five of about 45kDa, two with slightly higher (51kDa) and one with lower (37kDa) mass. In addition, this cluster included two tropomyosin protein species that greatly differed in mass (96 and 45kDa) and a MLC isoform of twice the typical mass (~43kDa). Finally, this cluster included serine threonine phosphatase 2A, Ca-dependent protein kinase 2, the small G-protein *cdc42* and the trimeric G-protein β subunit, all signaling proteins that are putatively playing a role in regulating the phosphorylation of MLC protein species. The overall change in abundance of proteins in this cluster indicate an increase with 0.12 μgL^{-1} lead at 12 h, but then a subsequent decrease with lead over the remaining exposure period. This pattern is illustrated best by *cdc42* (spot#205; Fig. 10A). However, both serine threonine phosphatase 2A and Ca-dependent protein kinase 2, which cluster with *cdc42*, do not show an interaction but instead a time effect (Fig. 9).

Cellular (non-muscle) tropomyosin (Tm) is mainly associated with actin [97]. While the typical Tm molecule is a dimer of about 35kDa, the mass of the two Tm isoforms of this cluster is greater (45 and 96kDa), but similar to the mass we observed in another invertebrate [98]. Interestingly, over 40 Tm splice variants are associated with the cytoskeleton of non-contractile cells, highlighting the potential for various actin regulatory functions [97]. Phosphorylation of Tm generates additional isoforms and implicates Tm in the regulation of stress fiber formation. In general, Tm regulates actin conformation and thereby actin-myosin interaction. This interaction is further regulated by MLC isoforms and their phosphorylation status [99]. Furthermore, both serine threonine phosphatase 2A and Ca-dependent protein kinase 2 might be directly involved in regulating the phosphorylation status of MLC protein species [100, 101]. However, potentially even more significant, further upstream, *cdc42*, a well-

characterized small G-protein, might affect actin-myosin interactions, as cdc42-controlled kinases affect MLC phosphatases, i.e. possibly phosphatase 2A, and thereby inhibit the formation of actin-myosin stress fibers [57]. Different types of stress fibers contain distinct combinations of actin, MHL and MLC as well as tropomyosin protein species and often resemble sarcomere-like contractile structures [102]. Stress fibers are induced in response to mechanical stress triggering the need for cells to oppose or compensate for these forces to maintain their structure [103]. The fact that several stress fiber-associated proteins first increased and then decreased with lead, specifically cdc42, which inhibits their formation [57], might suggest a switch from a lower rate early on to a higher rate of stress fiber formation with prolonged exposure to lead.

Cluster III includes three actins of high molecular mass, two myosin heavy chains (MHC) and paramyosin. While the MHC makes up the head as well as the rod of the thick filament (N- and C-terminus, respectively), paramyosin is only part of the rod in invertebrates [104-106]. The cluster is characterized by a decrease in abundance with lead at 12 h, with one MHC (spot#241) and paramyosin being significantly lower at either just 0.12 μgL^{-1} or both concentrations, respectively (Fig. 10C). In crustaceans, similar changes are an indication of changes in fiber type [107], possibly indicating shifts between different types of stress fibers [102].

Cluster IV included nine actins, with three isoforms representing fragments ranging in mass from 12-18kDa, two tubulins, low mass MHC, the actin-binding and membrane-anchoring filamin-C [108] and another isoform of cdc42 (Fig. 9). The cdc42 isoform (spot#283) was about equally abundant as the one from cluster II, but there were no significant pairwise comparisons between treatments (Fig. 10B and C).

The final cluster (V) included three actins (one of a mass of 100kDa), one tubulin, two Tm protein species, one MHC and four MLC (regulatory and essential isoforms of almost equal mass), calponin, an inhibitor of myosin ATPase and [109, 110] and the ADP-ribosylation factor, which is involved in vesicle formation [57]. The general pattern of changes in abundance indicates that most of the proteins in this cluster responded to lead strongly at 24 h and a little less strongly at 168 h, but not at 12 and 48 h. Several myosin regulatory and catalytic light and one heavy chain as well as two tropomyosin isoforms showed significant pairwise differences between the control and one or both of the lead concentrations at 24 h, and similarly at 168 h (Fig. 10C). These results suggest modification of the catalytic rate of actin-myosin interactions, possibly through changes of the phosphorylation of MLC isoforms. Interestingly, the

opposite pattern of changes occurred for cdc42 [spot#205], which affects MLC kinases, and several other signaling proteins at 168 h of exposure (cluster II).

In summary, lead affected the composition of actin-myosin contractile fibers in gill. Specifically, changes in MHC and MLC isoforms as well as tropomyosin suggest that the catalytic rates of the actin-myosin interaction changes with prolonged lead exposure. Several signaling proteins, including phosphatases and kinase as well as the small G-protein cdc42, may regulate these changes upstream.

CONCLUSION

Given that several of the proteins involved in the early changes to lead, e.g. *Mn*-SOD, CA and ferritin, carry metals as co-factors, it is possible that their replacement by lead is a major factor affecting the function of these proteins. If this is the case, e.g. reducing the rate of scavenging superoxide anions or of binding Fe^{2+} , it is possible that the net generation of ROS will increase and affect other cellular processes components, e.g. protein maturation in the ER, as this process is closely linked to the redox status of this compartment [85]. Further downstream, the increase in abundance of two α subunits of the proteasome with lead at 48 and 168 h suggests that protein chaperoning was inadequate to prevent protein denaturation (Figs 5 and 6).

A shift in abundance towards metabolic pathways that are recruited for ATP synthesis during stress or high ATP turnover, the phosphocreatine shuttle and glycolysis, indicates that lead disrupts energy homeostasis. Lead also increased the abundance of CA at 12 h, possibly affecting acid-base balance, which could be a secondary stress and a factor affecting a number of other cellular processes later in the exposure. Again, CA is a metal-binding protein (Zn) and replacing the metal is the most likely mechanism of how lead affects CA [93, 94].

Finally, cdc42, a key signaling protein of cytoskeletal dynamics, increased in response to $0.12 \mu\text{gL}^{-1}$ at 12 h and subsequently decreased at either one of the lead concentrations at 24, 48 and 168 h (Fig. 10A). Some of the changes that may be downstream from this signaling protein include possible modifications of the tail region of the thick filament, as indicated by a decrease of paramyosin and MHC (spot#241) with lead at 12 h (Fig. 10B). Elevated levels of four MLCs, among them catalytic isoforms, with lead followed at 24 and 168 h and might indicate additional changes

linked to the time course of cdc42 (Fig. 10D). Cdc42 was also found to increase with heat stress in gill of *Mytilus* [111].

Together, the results suggest that lead exposure will compromise the ability of *N. concinna* to respond to any additional stress, i.e. possible hypo-saline stress from an increase in melting ice, as several of the same processes, e.g. ER protein maturation and antioxidant proteins, are affected by both stressors in gills of mollusks [112]. It is thus likely that Antarctic marine invertebrates are particularly sensitive to a combination of stressors, such as increasing temperatures, pollution, and hyposaline stress, in the future.

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Author contributions

C.A.P., M.R.D.P., L.D. planned the experimental design and collected the samples. C.A.P. conducted the analysis of the gill samples at the Environmental Proteomics Lab at Cal Poly. C.A.P., M.H. and L.T. analyzed the data and wrote the manuscript.

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1020 16.

1021

1022 **Table 1:** Positive and negative loadings for principal component 1 and 2 of proteins significant for 12 hours exposition to lead (compare with
1023 Fig. 2A).
1024

Component Loading Rank	Principle Component 1		Principle Component 2	
	Protein (Spot ID)	Loandin Value	Protein (Spot ID)	Loandin Value
Positive Loadings for 12 hours				
1	(a) Ferritin (162)	1,81208	(c) Actin (103)	2,05570
2	Actin (285)	1,80768	eIF 3 (210)	1,98197
3	Carbonic anhydrase (195)	1,68094	Actin (114)	1,80019
4	β Tubulin (165)	1,63848	Tropomyosin (22)	1,78655
5	Pyruvate dehydrogenase E1 (198)	1,47488	Actin (122)	1,76191
6	Methylmalonate-semialdehyde dehy (211)	1,45828	Pyruvate dehydrogenase E1 (132)	1,69542
7	Actin (96)	1,44798	Actin (116)	1,67519
8	Actin (116)	1,39061	Actin (104)	1,57708
9	Calreticulin (109)	1,34643	Calreticulin (109)	1,56808
10	Mit. Malate dehydrogenase (272)	1,28358	Serine/threonine phosphatase 2 A (123)	1,48852
Negative Loadings for 12 hours				
1	(b) Carbonic anhydrase (197)	-1,97391	(d) Isocitrate dehydrogenase NADP (245)	-1,69668
2	Dihydrolipoyl dehydrogenase (254)	-1,79706	Carbonic anhydrase (204)	-1,65000
3	Pyruvate dehydrogenase E1 (14)	-1,69043	Myosin heavy chain (63)	-1,58034
4	Myosin heavy chain (241)	-1,64151	Protein disulfide isomerase (50)	-1,57609
5	Myosin regulatory light chain (189)	-1,63204	Actin (62)	-1,55668
6	Actin (36)	-1,45007	α -enolase (56)	-1,40424
7	Myosin heavy chain (179)	-1,43709	Phosphoglycerate kinase (192)	-1,40243
8	Myosin regulatory light chain (185)	-1,41410	Cdc42 (205)	-1,34103
9	Paramyosin (181)	-1,41350	β Tubulin (165)	-1,18197
10	Dihydrolipoyl dehydrogenase (244)	-1,34183	Carbonic anhydrase (195)	-1,16624

1025 **Table 2:** Positive and negative loadings for principal component 1 and 2 of proteins significant for 24 hours of exposition to lead (compare with
1026 Fig. 2B).

1027

Component Loading Rank	Principle Component 1		Principle Component 2	
	Protein (Spot ID)	Loading Value	Protein (Spot ID)	Loading Value
Positive Loadings for 24 hours				
1	(a) Actin (218)	1,92827	(c) Actin (10)	2,05474
2	Ferritin (162)	1,87892	Myosin heavy chain (9)	2,04783
3	Actin (65)	1,87017	Actin (137)	1,95309
4	Mn Superoxide dismutase (160)	1,86687	β Tubulin (46)	1,91382
5	Actin (95)	1,86657	Actin (64)	1,90539
6	78 kDa Glucose-regulated protein (20)	1,85595	Myosin regulatory light chain (119)	1,86439
7	Actin (76)	1,79823	Actin (112)	1,81726
8	Myosin heavy chain (179)	1,70460	Serine/threonine phosphatase 2 A (123)	1,80209
9	Actin (62)	1,70186	G protein β (292)	1,75428
10	Aldehyde dehydrogenase (264)	1,66479	Myosin heavy chain (241)	1,66965
Negative Loadings for 24 hours				
1	(b) Carbonic anhydrase (197)	-1,88689	(d) β Tubulin (169)	-1,94819
2	Actin (143)	-1,73734	Arginine kinase (274)	-1,74492
3	Actin (114)	-1,70659	Paramyosin (181)	-1,59505
4	Cdc42 (205)	-1,68771	Filamin-C (233)	-1,58944
5	Actin (116)	-1,64557	Myosin catalytic light chain (186)	-1,45870
6	Peroxidase 1/2 (247)	-1,43176	Cdc42 (283)	-1,43678
7	Mit. Malate dehydrogenase (272)	-1,38728	Actin (122)	-1,40147
8	α -enolase (56)	-1,08086	Triosephosphate isomerase (249)	-1,34969
9	Actin (115)	-1,06304	Triosephosphate isomerase (224)	-1,20213
10	Pyruvate dehydrogenase E1 (14)	-1,04308	Actin (70)	-1,19122

1028 **Table 3:** Positive and negative loadings for principal component 1 and 2 of proteins significant for 48 hours of exposition to lead (compare with
1029 Fig. 2C).
1030

Component Loading Rank	Principle Component 1		Principle Component 2	
	Protein (Spot ID)	Loading Value	Protein (Spot ID)	Loading Value
Positive Loadings for 48 hours				
1	(a) Actin (114)	1,79489	(c) Dihydrolipoyl dehydrogenase (254)	1,93543
2	Paramyosin (181)	1,73677	β Tubulin (169)	1,77021
3	Cdc42 (205)	1,62010	Phosphoglycerate kinase (192)	1,67557
4	Arginine kinase (237)	1,42303	Actin (143)	1,57960
5	Methylmalonate-semialdehyde dehyd. (264)	1,38784	Ferritin (162)	1,55136
6	Myosin catalytic light chain (184)	1,35179	β Tubulin (165)	1,46007
7	eIF3 (210)	1,34459	Calreticulin (43)	1,34515
8	Myosin heavy chain (179)	1,32972	Actin (122)	1,32463
9	Myosin regulatory light chain (189)	1,29358	Aldehyde dehydrogenase fam. 7 (242)	1,30168
10	Methylmalonate-semialdehyde dehydrog (211)	1,25447	Actin (297)	1,30147
Negative Loadings for 48 hours				
1	(b) Phosphorylase kinase α β (234)	-1,95837	(d) Actin (191)	-2,10195
2	Isocitrate dehydrogenase NADP (245)	-1,85437	Pyruvate dehydrogenase E1 (14)	-2,02865
3	Actin (218)	-1,81707	Proteasome β (305)	-1,93822
4	ADP Ribosylation fator (270)	-1,78843	Actin (36)	-1,81667
5	Pyruvate dehydrogenase E1 (132)	-1,74433	Myosin catalytic light chain (186)	-1,80209
6	Actin (116)	-1,63297	Ferritin (heavy) (66)	-1,74955
7	Ca-dependent protein kinase 2 (150)	-1,50677	Actin (62)	-1,69322
8	Mit. Malate dehydrogenase (272)	-1,49816	Actin (287)	-1,64958
9	Carbonic anhydrase (195)	-1,43945	Pyruvate dehydrogenase E1 (110)	-1,59030
10	Actin (137)	-1,40709	Actin (76)	-1,58938

1031 **Table 4:** Positive and negative loadings for principal component 1 and 2 of proteins significant for 168 hours of exposition to lead (compare with
1032 Fig. 2D).
1033

Component Loading Rank	Principle Component 1		Principle Component 2	
	Protein (Spot ID)	Loading Value	Protein (Spot ID)	Loading Value
Positive Loadings for 168 hours				
1	(a) Actin (95)	1,5955	(c) Actin (143)	2,0585
2	T-complex protein β (42)	1,5809	Phosphorylase kinase α β (234)	2,0316
3	Actin (36)	1,5724	78 kDa Glucose-regulated protein (20)	1,9043
4	β Tubulin (165)	1,5665	Ferritin (162)	1,6372
5	Mn Superoxide dismutase (160)	1,5632	Peroxidase 1/2 (247)	1,6149
6	Carbonic anhydrase (197)	1,5207	Tropomyosin (125)	1,5748
7	Actin (64)	1,4717	Carbonic anhydrase (195)	1,5445
8	Protein disulfide isomerase (50)	1,4279	α -enolase (56)	1,4599
9	Myosin regulatory light chain (119)	1,4178	Pyruvate dehydrogenase E1 (132)	1,4240
10	Ferritin (heavy) (188)	1,3907	Ca-dependent protein kinase 2 (150)	1,3333
Negative Loadings for 168 hours				
1	(b) Actin (104)	-1,6740	(d) Ferritin (316)	-1,9341
2	Myosin heavy chain (63)	-1,4921	Filamin-C (233)	-1,8132
3	Actin (122)	-1,4826	Dihydrolipoyl dehydrogenase (244)	-1,6989
4	Actin (137)	-1,4699	β Tubulin (169)	-1,5692
5	Actin (79)	-1,4534	Isocitrate dehydrogenase NADP (245)	-1,5393
6	Actin (52)	-1,4478	Methylmalonate-semialdehyde dehydroge (211)	-1,4885
7	Proteasome β (305)	-1,4102	Arginine kinase (237)	-1,4212
8	Actin (287)	-1,3614	Myosin heavy chain (179)	-1,4186
9	Carbonic anhydrase (204)	-1,3315	Pyruvate dehydrogenase E1 (14)	-1,3285
10	Actin (96)	-1,3308	Actin (76)	-1,3116

Lead (Pb)

Nacella concinna

2-DE

PCA

MALDI-TOF-TOF

antioxidant proteins

protein homeostasis

energy metabolism

cytoskeletal

Figure 1

Fig. 1

Figure 2A

12 hour exposure to lead (all significant proteins)

Fig. 2A

24 hour exposure to lead (all significant proteins)

Fig. 2B

48 hour exposure to lead (all significant proteins)

Fig. 2C

Fig. 2D

168 hour exposure to lead (all significant proteins)

Figure 3

Fig. 3

Figure 4

Fig. 4

Figure 5

Figure 6a

Fig. 6A

Figure 6B

Fig. 6B

Figure 7

Figure 8A

Fig. 8A

Figure 8B

Fig. 8B

Fig. 8C

Fig. 8D

Figure 9

Fig. 10A

Figure 10B

Fig. 10B

Fig. 10C

Figure 10D

Fig. 10D

Figure 10E

Fig. 10E