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Manuscript Draft

Manuscript Number: STOTEN-D-16-02131R1

Title: Sub-lethal cadmium exposure increases phytochelatin concentrations in the aquatic snail Lymnaea stagnalis

Article Type: Short Communication

Keywords: phytochelatin; cadmium; ecotoxicology; detoxification; mollusc

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Abstract: Phytochelatins are metal-binding metabolites found in almost all plant species and some animal groups, including nematodes and annelids, where they can play an important role in detoxifying metals such as cadmium. Species from several other taxa contain a phytochelatin synthase (PCS) gene orthologue, including molluscs, indicating they may have the potential to synthesize phytochelatins. However, the presence of a gene alone does not demonstrate that it plays a functional role in metal detoxification. In the present study, we show that the aquatic snail Lymnaea stagnalis produced both penta- and heptapeptide phytochelatins (i.e. phytochelatin-2 and phytochelatin-3), and their levels increased in response to sub-lethal levels of cadmium.

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Highlights

- Little is known about the role of phytochelatins in metal detoxification in animals
- We detected phytochelatins (PC_2 and PC_3) in a mollusc species, *Lymnaea stagnalis*
- Phytochelatins increased in *Lymnaea stagnalis* when exposed to cadmium
- Future research on phytochelatin responses in molluscs would be valuable

Sub-lethal cadmium exposure increases phytochelatin concentrations in the aquatic snail *Lymnaea stagnalis*

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23 Abstract

24 Phytochelatins are metal-binding metabolites found in almost all plant species and
25 some animal groups, including nematodes and annelids, where they can play an
26 important role in detoxifying metals such as cadmium. Species from several other
27 taxa contain a phytochelatin synthase (PCS) gene orthologue, including molluscs,
28 indicating they may have the potential to synthesize phytochelatins. However, the
29 presence of a gene alone does not demonstrate that it plays a functional role in metal
30 detoxification. In the present study, we show that the aquatic snail *Lymnaea stagnalis*
31 produced both penta- and heptapeptide phytochelatins (i.e. phytochelatin-2 and
32 phytochelatin-3), and their levels increased in response to sub-lethal levels of
33 cadmium.

34

35 Keywords

36 *Lymnaea stagnalis*, cadmium, metal pollution, phytochelatin, detoxification

37

38 1. Introduction

39 Phytochelatins (PCs) are non-ribosomal peptides, that are produced from glutathione
40 by the action of a phytochelatin synthase (PCS) enzyme (Cobbett and Goldsbrough,
41 2002). They have the structure $(\gamma\text{-GluCys})_n\text{Gly}$, with *n* so far observed to range from
42 2 – 4 for animal species. The free thiols on the cysteine residues can strongly chelate
43 metal and metalloid ions, such as cadmium and arsenic. PCs were first discovered in
44 fission yeast and in plants, but were furthermore shown to be present and functional in
45 an animal species: the nematode model organism *Caenorhabditis elegans*, where

knocking down the PCS enzyme made them hypersensitive to cadmium (Clemens et al., 2001; Vatamaniuk et al., 2001). PCS mutants also have an increased accumulation of cadmium compared to wild-type, indicating that PCS plays a role in metal detoxification (Essig et al., 2016).

Metallothioneins (MTs) are small proteins generally thought of as the prototypical thiol-rich metal-binding molecules in animals, and there are many studies on their responses and role in animal species (Amiard et al., 2006). MTs may not necessarily be the main players in detoxification, though, as knocking out the PCS gene in *C. elegans* makes it more sensitive to cadmium than does knocking out the two MT genes (Hughes et al., 2009). This has obvious and direct relevance for ecotoxicological research: perhaps PCs will turn out to be of major importance for many environmentally-relevant animal species. Studies on metal pollution which have not analysed PCs may turn out to give an incomplete picture (Bundy et al., 2014). Indeed, recent studies have shown direct responses of PCs to metal(loid) ions in animal species from additional phyla, including earthworms (Annelida) and sea squirts (Chordata) (Franchi et al., 2014; Liebeke et al., 2013). This, again, highlights the potential significance of PCs in the context of environmental pollution research, as they are found in phyla with members from the terrestrial, freshwater, and marine environments.

This still leaves one of the most ecologically relevant and speciose phyla unaccounted for – what about molluscs? Molluscs are also found in all three major environments (terrestrial, freshwater, and marine), and include key sentinel and monitoring species – many shellfish are of particular ecological and economic interest because they are filter-feeders and so can accumulate high levels of pollutants, and may be part of the human food chain. At least some mollusc species contain PCS

genes, including gastropod and bivalve species (Bundy and Kille, 2014), and PCS gene transcription was affected in response to metal ions in the mussel *Bathymodiolus azoricus* (Bougerol et al., 2015). However, gene expression is probably not a reliable reporter of actual changes in the functional end products, the PCs (Essig et al., 2016; Liebeke et al., 2013). In the present study we exposed a mollusc species, the freshwater snail *Lymnaea stagnalis*, to sub-lethal levels of cadmium (Cd), and used direct biochemical detection of PCs to determine the snails' response.

2. Materials and Methods

2.1 Culture and metal exposures

Lymnaea stagnalis cultures were established from a parasite-free culture established at the INRA centre (Rennes, France). The snails were acclimated (phase 1) in aquaria with tap water for two weeks and maintained under controlled conditions: light/dark photoperiod cycle of 16/8 hours; temperature of 20 ± 1ºC; pH of 7.5 ± 0.5; oxygen concentration > 6 mg L^{-1}; and ammonium concentration < 1.5 mg L^{-1}. One-third of the water was renewed weekly, and the snails were fed *ad libitum* with organic lettuce three times a week. The snails were then transferred into 1L glass vials for acclimation to the test conditions (phase 2, chronic exposure) until reproduction restarted. For phase 2, five concentrations of Cd (25, 50, 100, 200 and 400 µg Cd L^{-1}) were used, with six replicates per concentration and five snails (shell length 2.7cm±0.2) per replicate, plus a negative control with water only. The snails were exposed for 56 days, under the same light/dark cycle and physical-chemical conditions as described for the cultures. Full medium renewals were made twice a week (semi-static test) and snails fed *ad libitum* with organic lettuce. Survival and fecundity were also recorded at least twice a week, and dead snails were withdrawn

from the vials. Individual snails from each replicate vial were used for PC and Cd analysis.

2.2 Metal analysis

After the 56-day exposure, snails were lyophilized for 48h and pooled by replicate within each treatment. Then, for each replicate, the pool was ground and aliquots of the powdered tissue weighed for acid digestion. The tissue was digested in a mixture of trace analysis grade nitric acid (≥ 69%) + perchloric acid (67-72%), in a proportion of 7:1 v/v (1 ml for samples < 1mg and 2 ml for samples ≥ 1 mg), in a four-step temperature cycle (90ºC, 120ºC, 140ºC, and 160ºC) as described by Ardestani and van Gestel (2013). After cooling to room temperature, 10 ml of nitric acid (0.1M) was added to each digest. ICP-MS analysis was carried out with an i-CAP Qc instrument (Thermo Scientific, UK) in He-mode (KED-mode). External standard solutions were prepared from 10 mg/kg multi element standard AccuTrace® (AccuStandard®, New Haven, USA). Germanium was obtained from High-Purity Standards, USA and diluted to 5 µg/L using 0.1 % (v/v) nitric acid. The instrument was optimised for highest sensitivity under robust plasma conditions (high matrix insert in skimmer), with Ni-cones and Micromist nebulizer. He was used at 4.5 mL/min as the reaction cell gas. Integration time per isotope was 0.1 s, with 5 replicates each. Internal standard (Ge, 5 µg/L) was continuously added via a T-piece before the nebulizer. Blank samples were included in the analysis process, as well as certified reference material DOLT-3 (Dogfish liver) from National Measurement Standard of Research Council of Canada. Cd was below detection limits in all of the blank samples, and the CRM samples had a mean value of 24.2 mg Cd kg^{-1} (125% of the certified value), with a coefficient of variance of 35% (omitting one sample out of six for which no signal was obtained).

120 2.3 PC analysis

121 We ground each whole snail (approximately 1g wet weight) into 3 ml of methanol
122 (containing 9.8 mM tris(2-carboxyethyl)phosphine (TCEP) as a reducing agent) using
123 a pestle and mortar at liquid nitrogen temperatures, allowed the frozen powder to
124 thaw, and transferred the extract to a microcentrifuge tube. Following centrifugation
125 (5 min, 16,000g), the supernatants were derivatized with 60 µl of 266 mM *N*-ethyl
126 maleimide. We then analysed the phytochelatins PC_2 and PC_3 using a targetted LC-
127 MS-MS method, based on the approach of Liebeke et al. (2013). Briefly, samples
128 were injected onto an Agilent 1200 HPLC system coupled to a SCIEX 6500 Q-TRAP
129 mass spectrometer (AB SCIEX, Warrington, UK) operated in High Mass negative
130 mode. Chromatography was performed using a C18 column (100 x 3 mm, Ascentis
131 Express, Sigma-Aldrich, Poole, UK) and a gradient of 100% A (94.9% H_2O; 5%
132 acetonitrile; 0.1% formate) to 100% B (5% H_2O; 94.9% acetonitrile; 0.1% formate)
133 over 6 min. at 40^0C. The solvent flow rate was 250ul/min. PC_2 and PC_3 had a
134 retention time of 4.2 and 4.5 minutes, respectively. Data were acquired and analysed
135 with Analyst 1.4.2 software (Applied Biosystems). We used four mass transitions to
136 monitor each phytochelatin (Table 1). Collision cell exit potentials were set at -50v
137 and -13v respectively.

138 2.4. Bioinformatic analysis.

139 Unannotated snail PCS genes were identified from the TSA and WGS section of
140 NCBI by homology searching using BLASTX with the highly conserved N-terminal
141 domain from *C. elegans* PCS-1 (A5JYS0). The full length transcript was mapped to
142 the genome using Artemis (Rutherford et al. 2000), and exon/intron boundaries
143 manually validated. A maximum likelihood phylogenetic tree was generated using

144 MEGA 6.0 (Tamura et al., 2007) and shaded alignment of the conserved region
145 generated using BoxShade.
146

147 3. Results and Discussion

148 Three out of the five cadmium concentrations were high enough to kill some or all of
149 the snails during the exposure period: 100% of the snails died at the two highest
150 concentrations (200 and 400 µg Cd l^{-1}), and 90% died at the middle concentration
151 (100 µg Cd l^{-1}), with a calculated LC_{50} value of 78.2 ± 1.5 (S.E.) µg Cd l^{-1}. We
152 therefore carried out all further analyses (metal and PCs) only for the snails exposed
153 to sub-lethal cadmium concentrations (i.e. 0, 25 and 50 µg Cd L^{-1}). Gomot (1998)
154 reported low mortality during a 7-week exposure period, where 10 to 20% mortality
155 was observed under similar Cd concentrations used in the present study. In addition,
156 sub-lethal effects on egg development were observed at low concentrations (25 µg l^{-1}
157). Coeurdassier et al. (2003) were unable to obtain an LC_{50} for *L. stagnalis* exposed
158 to Cd at concentrations up to 320 µg L^{-1}, but the period of exposure was shorter (28
159 days). Such time dependency of effects is consistent with the known slow
160 toxicokinetics of Cd.

161 The exposure increased the snails' body burden of Cd. Tissue Cd
162 concentrations were below detection limits for the control group, and the 25 and 50
163 µL Cd L^{-1} exposure groups both had elevated Cd levels, 72 ± 11 mg/kg and 63 ± 15
164 mg/kg (mean ± SD, n=6) respectively. This suggested the possibility of analysing the
165 data as a two-class study, i.e. considering both the nominal exposure levels to have
166 effectively the same Cd level, since the two Cd exposure groups were not
167 significantly different from each other (t test, P = 0.25).

168 The *L. stagnalis* PCS gene has not been formally described. We identified and
169 annotated as single genomic loci (intron and exon structure) and full length transcript
170 in previously unannotated publically-available data (and have associated an updated
171 annotation with the GenBank entry) (Fig. 1A). Translation of the transcript
172 demonstrated that the encoded protein contains conserved key functional amino acids
173 (Fig. 1B). This reveals that a putative PCS is being actively expressed in central
174 nervous system tissue of *L. stagnalis.* This sequence enabled us to identify and
175 annotate a second full length PCS transcript from an orphan (unannotated) aquatic
176 snail *Biomphalaria glabrata* (GenBank: XM-013214798), also encoding all relevant
177 functional residues. A clustering analysis based on the conserved portion indicates
178 that snail PCS from *L. stagnalis* and *B. glabrata* cluster together, and then cluster
179 more loosely with other animal PCS sequences (including two other mollusc species),
180 but are distinct from plant and yeast PCS (Fig. 1C). The functionality of the *L.*
181 *stagnalis* PCS enzyme was not directly demonstrated by expressing it in a suitable
182 recombinant host, so it was important to check that changes in PCs were actually
183 measured and not some other compound. We are confident that our analytical
184 approach was specific for PCs, as four separate mass transitions were monitored for
185 all samples (Fig. 2).

186 PC_2 was present in all of the snail samples, including the controls, and PC_3 in all
187 but one of the control samples, where it was below the detection limit. Both PCs had a
188 highly non-normal distribution, but nonetheless there were clear differences in
189 response to Cd (Figure 3; Supplementary Table S1). PC_2 was present at a
190 concentration approximately an order of magnitude greater than PC_3. Both increased
191 in response to Cd, but although the relative concentrations of PC_2 remained higher
192 than PC_3, the Cd-induced increase in PC_3 was more statistically significant than for

193 PC_2. If the Cd-exposed samples were treated as a single group, as indicated by the
194 similarity in tissue Cd between the two treatment levels, then the increase for the "25
195 + 50 μg l^{-1}" group was significant at $P < 0.01$ for both PC_2 and PC_3 (Supplementary
196 Table S1). Furthermore, there were no significant differences between the PC levels
197 for the 25 μg l^{-1} vs. the 50 μg l^{-1} groups for either PC_2 or PC_3 ($P > 0.5$ for both).

198 Previous studies of *L. stagnalis* detoxification responses to Cd and other
199 potentially toxic metals focussed on MTs, in both laboratory and field studies
200 (Gnatyshyna et al., 2011; Leung et al., 2003; Ng et al., 2011). To our knowledge, no
201 other study has directly analysed PC responses in any mollusc species. Our current
202 study is a preliminary observation, since we have not yet fully demonstrated that PCs
203 have a functional role in metal detoxification in *L. stagnalis*, although we do consider
204 it is the most probable explanation. Of course, this kind of functional demonstration is
205 much harder when working with non-model organisms like *L. stagnalis* compared to
206 *C. elegans*, for example, where it is relatively straightforward to obtain a mutant
207 strain, or modulate gene activity by RNA interference. It is no coincidence that *C.*
208 *elegans* is the animal species for which there is currently the greatest degree of
209 knowledge about PC function. Nonetheless, one can still build up a mechanistic
210 understanding of responses to toxins in non-model organisms, even though it may
211 require a greater range and quantity of data (Bundy et al., 2008).

212 The growing body of evidence that PCs are metal-responsive in multiple
213 animal species argues in favour of the potential importance of PC synthesis in
214 invertebrate responses to toxic metals (in at least some phyla). Phytochelatins are
215 more protective than MTs in *C. elegans*, the sole animal species for which a direct
216 comparison has yet been made (Hall et al., 2012; Hughes et al., 2009). Some patterns
217 are starting to emerge: in the present study, PC_2 had much higher concentrations than

218 PC_3, but the increase in PC_3 was more statistically significant. Both of these
219 observations are consistent with results from other animal species (earthworms and
220 nematodes) (Hughes et al., 2009; Liebeke et al., 2013).

221 *L. stagnalis* contains a PCS orthologue, but the presence of a gene alone does
222 not demonstrate that it plays a functional role in metal detoxification. It is not even
223 sufficient to show that the gene product is potentially active: the PCS from the
224 platyhelminth *Schistosoma japonicum* can synthesize PCs when heterologously
225 expressed in fission yeast, but *S. japonicum* itself apparently does not produce PCs in
226 response to Cd (Ray and Williams, 2011; Rigouin et al., 2013). Gene expression
227 analysis adds another piece of evidence, but again may well be insufficient to show
228 functionality in animal species. In plants, PC production is not regulated by
229 transcription: the substrate for the PCS enzyme is glutathione coordinated by metal
230 ions, so any entry of free metal ions into the cells will result in formation of GSH
231 complexes and rapid PC synthesis (Cobbett and Goldsbrough, 2002). There is much
232 less information about the regulation of animal phytochelatins. Liebeke et al. (2013)
233 found no association between PCS gene expression and PC production in response to
234 arsenic in the earthworm *Lumbricus rubellus*. Similarly, low levels of Cd did not
235 result in upregulated *pcs-1* gene expression in *C. elegans* (Cui et al., 2007). Other
236 studies on different earthworm species had some intriguing hints about possible
237 regulation, but because they did not measure PC levels directly, the relationship to
238 gene expression could not be determined. Brulle et al. (2008) saw differences in PCS
239 expression for *Eisenia fetida* at low but not high Cd concentrations, whereas Homa et
240 al. (2015) saw differences for *Eisenia andrei* for essential (Cu, Zn) but not non-
241 essential metals (Cd, Pb).

In conclusion, our current study demonstrates that the *L. stagnalis* PCS gene is functional, as shown by synthesis of PCs in response to cadmium. A full understanding of the role of PCs in metal detoxification in *L. stagnalis* and in other molluscs will require considerable additional research. In particular, nothing is currently known about the kinetics of the PC response; the interaction (if any) of PCs with MTs; the role (if any) of gene transcription; and the responses to metals and metalloids other than Cd. Direct analysis of PCs is essential for future studies of animals with PCS genes.

4. Acknowledgements

We thank Virginie Ducrot and INRA for providing the snails and the opportunity to participate in the reproduction toxicity test design for *Lymnaea stagnalis*. This study was partially supported by a grant to SFG (PTDC/AAC-AMB/117178/2010). CESAM (UID/AMB/50017/2013) also received financial support through FCT/MEC (through national funds) and the co-funding by the FEDER (POCI-01-0145-FEDER-00763), within the PT2020 Partnership Agreement and Compete 2020.

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355

6. Figure legends.

Figure 1. Gene structure, organisation and evolutionary conservation of *Lymnaea stagnalis* phytochelatin synthase. Panel A provides an illustration of the complete loci encoding the *L. stagnalis* PCS gene, located on draft genome assembly gLs.1.0.scaf01115_contig_7 (Gb:FCFB01038856; Exon 1:1405-1572, Exon 2:2333-2438, Exon 3:2653-2825, Exon 4:3336-3436, Exon 5:4159-4277, Exon 6: 4759-4903 and Exon 7:5293-6121). Evidence for the transcript was provided by a full gene assembly from Transcript Shotgun assembly performed from *Lymnaea* Central Nervous System (FX186822: CDS: 154-1866) (Sadamoto et al., 2012). Panel B shows an alignment of the PCS conserved functional region from two snail *L. stagnalis* PCS (Ls - FX186822) and *Biomphalaria glabrata* mRNA (Bg - XM_013214798.1); representatives of two additional Mollusca PCS, *Lottia gigantea* (LgPCS - ESP03342 and *Crassostrea gigas* (CgPCS - EKC27807); a PCS from the nematode *Caenorhabditis elegans* (Ce1a - A5JYS0); and two plant isoforms from *Arabidopsis thaliana* (At1 - Q9S7Z3, At2 - Q9ZWB7). Residues showing >50% conservation shown with a black background, and with those showing functional conservation shown in grey. Residues identified as essential for function are indicated by arrows. Panel C shows a maximum likelihood phylogenetic tree based on the PCS functionally conserved region shown aligned in Panel B.

Figure 2. Phytochelatins were detected specifically by LC-MS-MS, with very low background. The figure shows a representative chromatogram of A: the phytochelatin-2; and B: the phytochelatin-3 peak (both represented by four separate mass transitions) in a single sample extracted from an exposed snail.

381 **Figure 3**. Phytochelatins are increased in *Lymnaea stagnalis* as a result of Cd
382 exposure. Diamonds show mean (of log-transformed data) and 95% confidence
383 intervals (diamond vertices). A: phytochelatin-2; B: phytochelatin-3. Comparisons
384 between the groups are based on one-way Mann-Whitney tests; * corresponds to $P < 0.05$, and ** corresponds to $P < 0.01$. Exact values given in supplementary Table S1.

Table 1. Mass spectrometer parameters for detection of phytochelatins as *N*-ethyl maleimide derivatives. Q1 and Q3 refer to the targeted parent and daughter ions, respectively, with four different mass transitions monitored for PC_2 and PC_3.

Q1 Mass (Da)	Q3 Mass (Da)	Dwell time (ms)	Collision energy (eV)
790.2	661.4	50	30
790.2	358.3	50	45
790.2	304.3	50	45
790.2	267.3	50	70
1147.2	1018.4	50	45
1147.2	586.4	50	60
1147.2	558.4	50	70
1147.2	358.1	50	70

Figure 1

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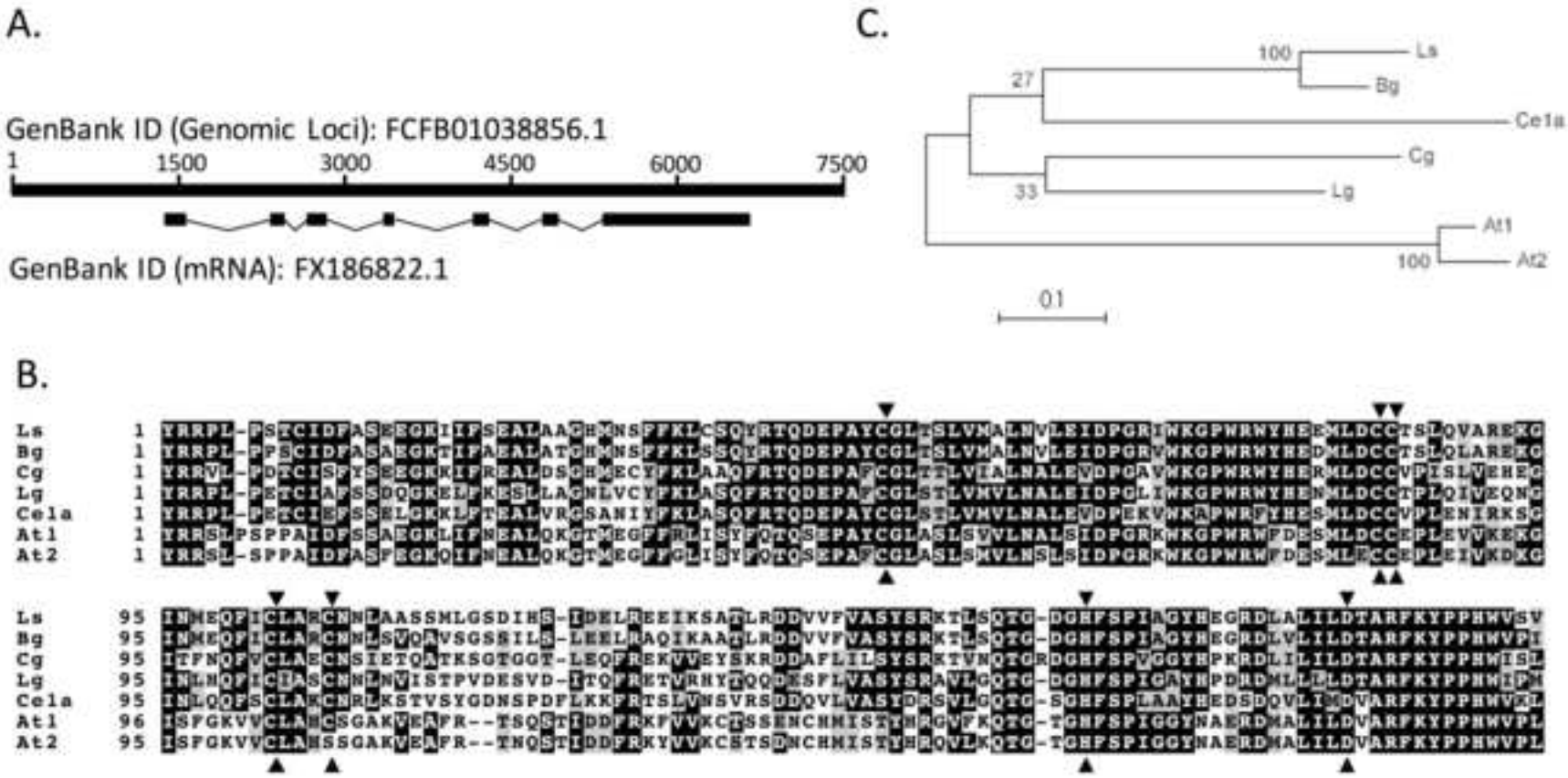

Figure 2

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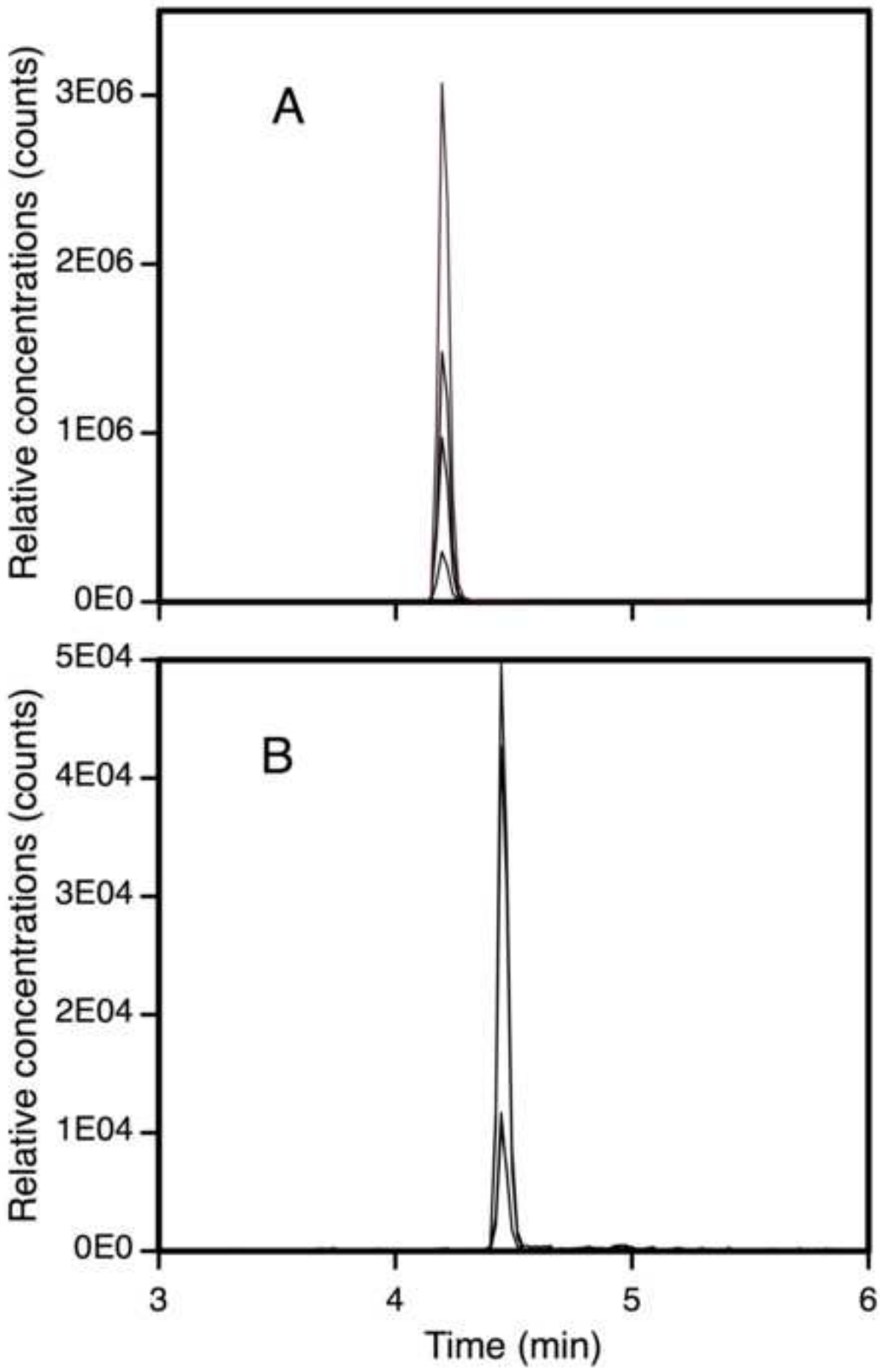

Figure 3

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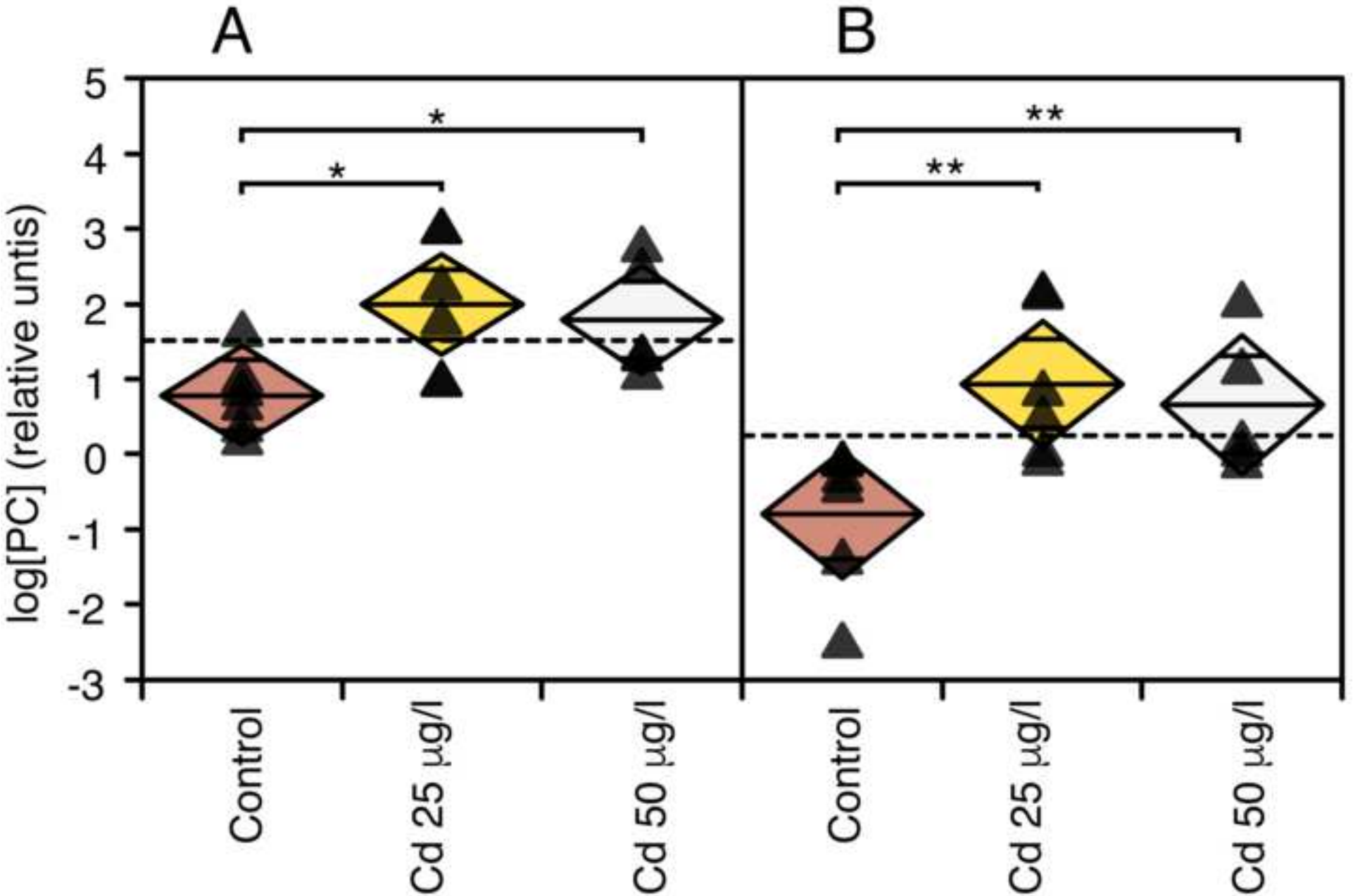

Table S1

Click here to download Supplementary material for on-line publication only: Table S1.docx