Ecotoxicology and Environmental Safety 138 (2017) 223–230

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Changes in fatty acid metabolism induced by varied micro-supplementation with zinc in snails *Helix pomatia* (Gastropoda Pulmonata)☆

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ARTICLE INFO

Keywords:
Zinc
Chelates
Fatty acid
Snail
Biomarker

ABSTRACT

We analyzed the changes in the profile of fatty acids (FA) in the foot tissues and hepatopancreas (HP) of snails *Helix pomatia* exposed to five microdoses of zinc (0.1, 0.25, 0.5, 0.75, or 1 mg/l) administered in the form of a pure salt solution and in the form of EDTA and lysine chelates. Selection from a pool of 56 fatty acids analyzed in snail tissues yielded a set of 12 biomarker acids undergoing significant changes in contact with toxic substances. The selection criteria included the greatest percentage among the FA profile and their significant role in physiological processes. The proposed palette of acids of the biomarker FAs comprised C16:0; C18:0; C23:0; C18:1 n-9; C20:1 n-9; C18:2 n-6; C18:3 n-3; C20:2; C20:4 n-6; C20:5 n-3; C22:4 n-6; and C22:5 n-3, and saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs), determined separately in the foot tissues and hepatopancreas. The significant ($p = 0.01$) influence of the dose as well as the source of the zinc on its' concentration in the tissues and on changes in the fatty acid profiles. Among the three zinc forms administered to the snails, the highest bioaccumulation of zinc in both tissues was noted in the group receiving the Zn-EDTA chelate. The content of PUFAs increased as the supplementation with zinc increased up to 0.75 mg/l, but at 1 mg/l, the share of these FAs began to decrease. This trend was observed in both analyzed tissue types - foot and hepatopancreas. The dose of 1 mg Zn/l might be considered as a threshold dose above which the saturation of FAs increases. The results proved that determination of FA profile in snails can be used in ecotoxicological research as a reliable test of the effect of trace doses of stressors. The micro-supplementation of the mollusks diet with zinc is an example of a non-routine approach to issues connected with both diet and toxicology.

1. Introduction

When the keywords: mollusks, gastropods, and metals appeared together in the literature, the idea that joined them was to use of mollusks as bioindicator organisms in ecotoxicology (Beeby and Richmond, 2002, 2011; Downs et al., 2001; Jordaens et al., 2006a; Kowalczyk-Pecka et al., 2015; Liang et al., 2004; Menta and Parisi, 2001). Mollusks are sensitive to minimal changes in food parameters, which due to their rapid metabolism in these animals are instantly reflected in numerous biochemical factors (Misra et al., 2002; Notten et al., 2006; Pirini et al., 2007; Radwan et al., 2010a, 2010b). Research has been aimed at identifying negative functional and physiological changes caused by environmental stressors such as metals (Chandran et al., 2005; Pirro and Marshall, 2005). There have also been numerous descriptions of the ability of mollusks to accumulate and magnify various chemical compounds in their soft tissues and to incorporate them in their shells, in both aquatic and terrestrial ecosystems (Capinera and Dickens, 2016; Jordaens et al., 2006b). However, there have been no reports of subthreshold doses of stressors inducing processes in mollusks. Metals are regarded as pollutants when they are present in the environment in amounts sufficient to stimulate perceptible and measurable changes in living organisms (Vijayavel et al., 2007). The final level of accumulation of metals is influenced by the length of the period of administration, dosage, availability, form of administration, the age of the animal, breeding conditions, other feed components, and the type of tissue analyzed. Land snails are first-order consumers and therefore they can serve as model examples of a rapid physiological response to changing conditions in the natural or breed-

☆ This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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http://dx.doi.org/10.1016/j.ecoenv.2016.12.033
Received 18 July 2016; Received in revised form 15 December 2016; Accepted 27 December 2016
Available online 09 January 2017

ing environment, including diet. Any changes in cellular processes, including structural and functional changes, which can be shown in a specific way, can be biomarkers (Sampedro et al., 2006). One such biomarker of the physiological state of gastropods is changes in the fatty acid profile.

The hypothesis of the research proposes that microdoses of zinc alter the fatty acid profile in snails, particularly the progression and regression of polyunsaturated fatty acids that can be used in routine ecotoxicological tests and can have potential practical application in snail breeding. For this purpose, we attempted to explore the effect of five microdoses of zinc administered in three forms: inorganic (pure salt solution) and organic (EDTA and lysine chelates) as a dietary supplement for *Helix pomatia* L. on chosen biomarker fatty acids determined in the foot tissues and hepatopancreas of the snails.

2. Material and methods

2.1. Experiment design

A naturalized laboratory population of *Helix pomatia* L. snails was maintained at the Department of Zoology, Animal Ecology and Wildlife Management of the University of Life Sciences in Lublin on a diet prepared according to Ligaszewski et al. (2008) with slight modification; i.e. without soybean oil, which could affect the natural profile of fatty acids (FA). Adult snails with a well-defined lip on the shell, weighing 23.0 ± 1.0 g and having shells 40.0 ± 1.0 mm in diameter, were sampled from the laboratory population. After they had been thoroughly rinsed with water, the snails were placed individually in perforated plastic containers (15 × 15 × 5 cm) and then transferred to a phytotron chamber (BIOGENET; 20 °C, RH = 90%, photoperiod 18 h L/6 h D) for further experiment. The experiment involved 170 snails divided into 17 groups, 10 snails in each.

To determine the possible influence of the phytotron chamber environment on the FA profile of the snails, an initial control group (Initial) was also included in the experiment. The snails from the initial group were sampled from the laboratory population and left without food for 48 h to clear the digestive tract, and then frozen at −70 °C for further biochemical analysis.

The snails from the remaining 16 groups were kept in the BIOGENET for 12 weeks. The animals were fed twice a week with a medium prepared according to Laskowski and Hopkin (1996) with slight modification (without any fungicide, which is a xenobiotic and might affect the FA profile). The medium contained 1 g of agar (Difco), 3 g of dried and powdered carrot root (*Daucus carota* L.), 0.5 g of milk powder (SM Siedlce) + 0.5 g wheat bran, and 0.01 g CaCO3 (BDH Ltd, UK); it was suspended in double-distilled water to obtain 100 ml of medium. A 15 ml volume of the medium was poured into Petri dishes and left to solidify before individual feeding. This volume of the medium was estimated to be consumed completely and was therefore optimal. The snails from the 15 experimental groups were exposed to zinc administered each time the medium was given to the snails. Five groups of snails received *per os* (with a pipette) 10 µl of a solution containing Zn^{2+} ions ($ZnCl_2$, POCH S.A.) at concentrations of 0.1, 0.25, 0.5, 0.75, or 1 mg/l redistilled H_2O (groups designated as Zn 0.1, Zn 0.25, Zn 0.5, Zn 0.75, and Zn 1.0, respectively). Another five groups of snails received *per os*, also twice a week, 10 µl of a solution containing a zinc EDTA chelate (POCH S.A.) at concentrations of 0.1, 0.25, 0.5, 0.75, or 1 mg/l redistilled H_2O (groups designated as Zn + EDTA 0.1, Zn + EDTA 0.25, Zn + EDTA 0.5, Zn + EDTA 0.75, and Zn + EDTA 1.0, respectively). Further five groups received, also twice a week, a solution containing a zinc lysine chelate (POCH S.A.) at concentrations of 0.1, 0.25, 0.5, 0.75, or 1 mg/l redistilled H_2O (groups designated as Zn + Lys 0.1, Zn + Lys 0.25, Zn + Lys 0.5, Zn + Lys 0.75, and Zn + Lys 1.0, respectively). The laboratory control group of snails (Control) received the medium twice a week, with the addition of 10 µl of pure redistilled water (*per os*). After 12 weeks, the snails from all groups were left without food for 48 h to clear the digestive tract and then frozen at −70 °C for further biochemical analysis.

2.2. Zinc analysis in the tissues

For metal analysis, tissue samples (foot and hepatopancreas – HP) were dried for 18 h at a temperature of 80 °C until a solid dry mass was obtained. The same procedure was used for the medium with the agar supplement given to the snails. Each sample was weighed, placed in an amount of approx. 200 mg in 5 ml of a mixture of nitric acid (65%) and perchloric acid (70%), and heated in a heating block at 80 °C for 2 h and 2.5 h at 210 °C until mineralization of the sample. The cooled suspension was filtered (Whatman 541 filter) and made up to 25 ml with deionized distilled water. After the samples were digested, zinc content was analyzed using atomic absorption spectrometry with a Unicam 939/959 apparatus. Results were given in µg per g dry weight of foot and hepatopancreas tissue.

2.3. Obtaining fatty acid methyl esters

A pool of 56 fatty acids was analyzed in the foot and hepatopancreas tissues of the snails. In order to control the dietary input of FA, the medium FA profile was analyzed as well (Table S1). After initial preparation and lyophilization of the analyzed material, lipids were extracted from the medium, as well as from the hepatopancreas and foot of each snail with a Soxhlet extractor (VELP SCIENTIFICA ser 148 Solvent Exractor). 50 mg of lipids were collected to obtain fatty acids. Fatty acid esters were obtained according to PN-EN ISO 12966-1:2015-01 and PN-EN ISO 12966-2:2011 standards and AOAC Official Method 969.33 (1969). The ester samples were analyzed using a Varian 3800 gas chromatograph with a FID detector and a CP-Wax 52CBWCOT Fused Silica capillary column, 60-m length and with an inner diameter of 0.25 mm. The initial temperature for the analysis was 120 °C for 5 min and the final temperature was 210 °C. The injector temperature was 260 °C and the detector temperature was 260 °C. The hydrogen flow rate was 30 ml/min, air flow-300 ml/min, and helium flow-1.4 ml/min. The volume of the injected sample was 1 µl. The results for the percentage content of fatty acids in the sample were obtained using Star GC Workstation Version 6.30.

The FAs proposed as biomarkers for further studies were selected in two steps. Initially, 21 FAs that were mostly affected by the zinc treatment were chosen from the pool of 56 FAs (Table S2-S3). Next, to simplify the methodology, 12 FAs were selected. The selection criteria included the greatest percentage among the 21 FAs chosen in the first step and their significant role in physiological processes. The final set of the biomarker FA comprised C16:0; C18:0; C23:0; C18:1 n-9; C20:1 n-9; C18:2 n-6; C18:3 n-3; C20:2; C20:4 n-6; C20:5 n-3; C22:4 n-6; and C22:5 n-3. The sums of saturated fatty acids (SFAs), iso-SFAs, anteiso-SFAs, monounsaturated (MUFAs), polyunsaturated (PUFAs), n-6, and n-3 fatty acids and their ratios were taken into consideration as well.

2.4. Statistical analysis

All the data were analyzed with Statistica software ver. 10 (StatSoft, 2011, License No. AXAP307F818708FA-K). The normality was assessed using the Kolmogorov-Smirnov test, and the Levene's homogeneity of variance test was applied to examine the equality of variances. Two-way ANOVA was used to assess the effect of the dose (0.1, 0.25, 0.5, 0.75 or 1 mg/l) and the source of the microelement (Zn chloride, Zn-EDTA, and Zn-lysine) and their interaction on the content of each FA. One-way ANOVA and Tukey's multiple range tests were performed to compare all the experimental groups and the control one. Pearson correlation coefficients between the experimental factors and the FA content were also calculated at a significance level of $p \leq 0.01$.