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ORIGINAL ARTICLE

Tissue metallothionein response in the Japanese quail associated with exposure to cyanobacterial biomass, lead and the Newcastle disease virus

Veronika Kovacova, Vladimir Piacek, Ehdaa Eltayeb Eltigani Abdelsalam, Hana Bandouchova, Tomas Heger, Katerina Kobelkova, Jana Sedlackova, Frantisek Vitula, Jiri Pikula

Department of Ecology and Diseases of Game, Fish and Bees, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic

Correspondence to: Mgr. Ing. Veronika Kovacova, PhD.
Department of Ecology and Diseases of Game, Fish and Bees, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackeho tr. 1946/1, 612 42 Brno, Czech Republic.
TEL: +420541562475; E-MAIL: kovacovav@vfu.cz

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Abstract

OBJECTIVES: Here we tested the hypothesis that multiple toxic and infectious stressors combine in their adverse effects to produce higher tissue responses of metallothioneins (MTs) in birds.

METHODS: We used Japanese quails as a model avian species. The study is based on data obtained from single and combined exposures of Japanese quails to cyanobacterial biomass containing microcystins, lead and a live Newcastle disease vaccination virus. Eight groups of 5 birds were exposed to single, double and triple combinations of these stressors and compared with controls. Birds were euthanized after the 30-day exposure to collect brain, liver, kidney, and pectoral muscle for MTs measurement.

RESULTS: Baseline levels of MTs differed in avian tissues. The gradient of MTs in control quails was pectoral muscle < liver < brain < kidney. Double and triple exposures induced higher levels of MTs. While increases of MTs were driven mainly by dietary exposure to cyanobacterial biomass and/or lead, Newcastle disease vaccination induced the least response. Induction of brain MTs was dominated by exposure to lead. Patterns of MTs responses were similar in the liver and pectoral muscle as well as in the kidney and brain.

CONCLUSIONS: Understanding better responses of birds to oxidative stress induced by toxic and infectious stressors may have implications for avian conservation issues and disease risk assessment.

INTRODUCTION

Free-ranging wildlife is under pressure of both infectious agents and noninfectious stressors (Guitart *et al.* 2010; Bandouchova *et al.* 2018). Exposure to stressors results in a deterioration in health and wildlife mortality. While sublethal adverse effects and sporadic deaths may go undetected (Vyas 1999), new emerging issues, mass mortality events and population declines in species of conservation concern attract great attention (Fry 1995; Blehert *et al.* 2009). Moreover, combined exposure to multiple stressors is a realistic environmental scenario (Kortenkamp *et al.* 2007; Bandouchova *et al.* 2011).

To be more specific and examine the actions of important stressors out of the enormous diversity of infectious agents, natural toxins, pesticides, industrial chemicals and other environmental contaminants endangering birds, we selected cyanotoxins, lead and the Newcastle disease virus.

Cyanobacteria in many aquatic ecosystems are capable of producing a variety of toxic cyanotoxins with hepato-, neuro-, cyto- and dermato-toxic potential. Mass deaths in several avian species were attributed to the long-term foraging of birds at sites disturbed by cyanobacterial water blooms (Alonso-Andicoberry *et al.* 2002; Krienitz *et al.* 2002). Surprisingly, only sub-lethal effects were detected in experimental birds who were administered orally with both environmentally relevant and much higher doses of microcystins (MCs) in a cyanobacterial-biomass-supplemented diet (Skocovska *et al.* 2007; Damkova *et al.* 2009; Peckova *et al.* 2009; Damkova *et al.* 2011; Kral *et al.* 2012).

Elevated levels of toxic metals are among globally important environmental and health concerns (Nriagu 1988). Lead has been shown to impair general health, reproduction and embryo development, immune functions and blood physiology in birds (Fair & Ricklefs 2002). Ingestion of lead shot is the primary source of exposure and poisoning in most avian species associated with the use of lead ammunition (Gangoso *et al.* 2009). Ingested lead enters the bloodstream and then accumulates in the organs like liver, kidney, brain, bones and muscles (Pikula *et al.* 2013).

As a widespread agent, the Newcastle disease virus is capable of causing substantial mortality in wild birds. However, virus isolates show variable virulence and there are also differences in host species susceptibility (Kim *et al.* 2007).

What do actions exerted by stressors such as cyanotoxins, heavy metals and infectious agents have in common? First, all are capable of causing sublethal effects depending on the dose and/or isolate virulence. Second, a similar mode of action (Kortenkamp *et al.* 2007) can be identified, i.e., the oxidative stress associated with both kinds of poisonings and immune responses to infection (Mateo *et al.* 2003; Paskova *et al.* 2008; Costantini & Moller 2009). Third, the tissue response to exposure to stressors is in the form of metallothioneins (MTs), because these play a role in both the homeostasis and detoxification of the organism following abnormal exposure to toxic substances and in cell protection from oxidative stress (Pikula *et al.* 2010; Kral *et al.* 2015).

Here, based on the above-mentioned similarities, we proposed an avian model to test the hypothesis that single, double and triple exposures to stressors such as cyanobacterial biomass, lead and immune challenge by the live Newcastle disease vaccination virus result in different tissue metallothionein responses. Data concerning the clinical signs, mortality, haematology, biochemistry, antibody response, histopathology, body weight changes and toxin accumulation from this experiment have already been evaluated and published (Pikula *et al.* 2010; Paskova *et al.* 2011). Understanding better the responses of birds to toxic and infectious stressors may be applicable to conservation issues and disease risk assessment.

MATERIAL AND METHODS

Ethics statement

The study was performed in line with the OECD Guideline for the testing of chemicals 205-Avian Dietary Toxicity Test (OECD 1984). Experiments complied with the laws for the protection of animals against cruelty and were approved by the Ethical Committee of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. Permit No. 9221/2009-30 was issued by the Ministry of Education, Youth and Sports of the Czech Republic.

Experimental design

We used the experimental design described in Pikula *et al.* 2010. Briefly, four month old male Japanese quails (average weight 219 g) were used as an avian model of responses to exposure to multiple stressors. The study is based on data obtained from 30-day single and combined exposures of Japanese quails to cyanobacterial biomass, lead and a live Newcastle vaccination strain. A total of 40 birds were divided on a random basis into 8 groups of 5 individuals, i.e. control (C), cyanobacterial biomass-exposed birds (B), lead-exposed birds (Pb), cyanobacterial biomass-exposed+lead-exposed birds (B+Pb), Newcastle-vaccinated control birds (V), cyanobacterial biomass-exposed+Newcastle-vaccinated birds (B+V), lead-exposed+Newcastle-vaccinated birds (Pb+V), and cyanobacterial biomass-exposed+lead-exposed+Newcastle-vaccinated birds (B+Pb+V). Birds were held individually in standard laboratory cages for birds (floor area 1500 cm^2/bird), at room temperature, 12 h of light per day, and were provided with commercial feed and drinking water *ad libitum*.

The daily dose of cyanobacterial biomass was prepared as described in Skocovska *et al.* (2007): cya-

nobacterial biomass (1.92×10^9 cells, 83.46 mg dry weight), microcystin structural variants (15.36 µg MC-RR, 12.70 µg MC-YR, 17.98 µg MC-LR, 46.044 µg sum of MCs) for 30 days. Birds from groups B, B+Pb, B+V and B+Pb+V were fed twice a day using a crop probe to reach the daily dose of 10 mL of cyanobacterial biomass. Birds from groups C, Pb, V and Pb+V were administered twice a day with 5 mL of control water. A total of six 3.5 mm pellets (containing from 1.38 to 1.59 g lead per bird) were used to expose each bird to the toxic metal (individuals from groups Pb, B+Pb, Pb+V and B+Pb+V). Lead shot (Sellier & Bellot, Vlašim, Czech Republic) were inserted into the crop on day 0 of the experiment (see Pikula *et al.* 2010). The third stressor experimental birds were exposed to a live Newcastle disease vaccination strain (Avipest lyof. a.u.v. containing Paramyxovirus pseudopestis avium phyl. La Sota min. 106.0 EID_{50} per dose, Mevak a.s., Nitra, Slovakia). Each individual from groups V, B+V, Pb+V and B+Pb+V were vaccinated into the nostrils at the beginning of the experiment. The surviving birds were euthanized on day 30 by decapitation and selected organs (brain, liver, kidney, pectoral muscle) were collected for the measurement of cyanotoxin, heavy metals and metallothioneins. This study shows only the tissue metallothionein responses. Cyanotoxin and heavy metal levels in tissues from this experiment have already been published (Pikula *et al.* 2010).

Biochemical methods

The tissue levels of MTs were determined by using the absorptive transfer stripping technique with differential pulse voltammetry, Brdicka reaction (electrochemical detection) as has been detailed previously (Adam *et al.* 2007). MTs levels were measured as µM per gram of fresh weight mass.

Statistical analysis

Statistical analyses were performed with Statistica for Windows® 10.0 (StatSoft, Tulsa, OK, USA) and included the testing of data normality and homogeneity of variances by the Kolmogorov-Smirnov test or Levene´s test. Treatment groups were compared by one-way analysis of variance (ANOVA) and post-hoc LSD test. Values $p<0.05$ or $p<0.01$ were considered significant for all tests.

RESULTS

As shown in Figures 1 to 4, MTs levels in the healthy control Japanese quails were tissue-specific, with the lowest and highest values in the pectoral muscle and kidneys, respectively. MTs levels of vaccinated controls (i.e. group C+V) were as low as those of the control group C in all tissues. Comparing the pattern of MTs induction by single, double and triple exposures to the studied stressors, responses in the liver and pectoral muscle were similar (cf. Figures 1 and 4). Likewise, the tissue from the kidney responded to multiple stressors in a pattern similar to the brain (Figures 2 and 3). Considering the liver (Figure 1), double and triple exposures to stressors responded with a higher level of MTs induction. Significant elevations of kidney MTs were observed in the following groups of exposure: Pb,

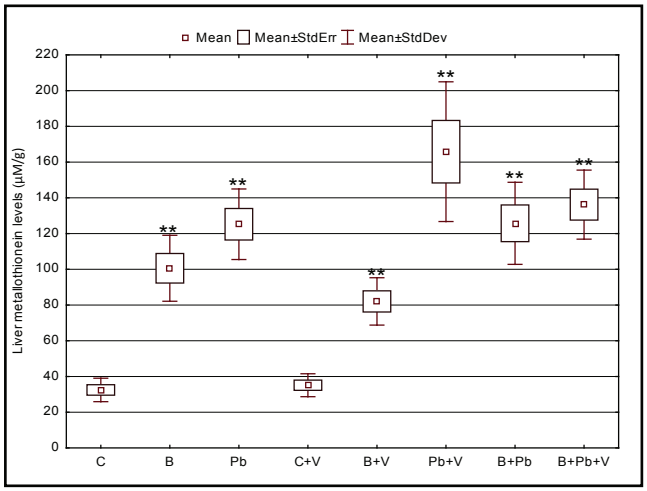

Fig. 1. Liver metallothionein levels in Japanese quail groups on day 30 of exposure. Groups C = control, B = exposure to cyanobacterial biomass, Pb = exposure to lead, C+V = vaccinated control, B+V = exposure to cyanobacterial biomass and Newcastle vaccination, Pb+V = exposure to lead and Newcastle vaccination, B+Pb = exposure to cyanobacterial biomass and lead, B+Pb+V = exposure to cyanobacterial biomass, lead and Newcastle vaccination. Measured in fresh weight tissue, n=5 in all groups, $^{**}p<0.01$.

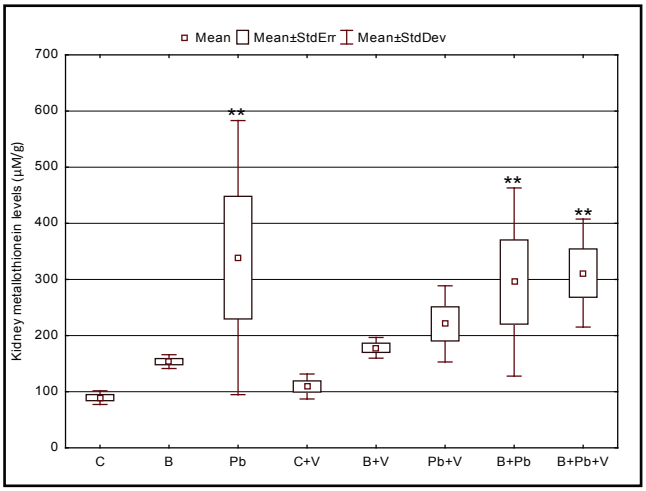

Fig. 2. Kidney metallothionein levels in Japanese quail groups on day 30 of exposure. Groups C = control, B = exposure to cyanobacterial biomass, Pb = exposure to lead, C+V = vaccinated control, B+V = exposure to cyanobacterial biomass and Newcastle vaccination, Pb+V = exposure to lead and Newcastle vaccination, B+Pb = exposure to cyanobacterial biomass and lead, B+Pb+V = exposure to cyanobacterial biomass, lead and Newcastle vaccination. Measured in fresh weight tissue, n=5 in all groups, $^{**}p<0.01$.

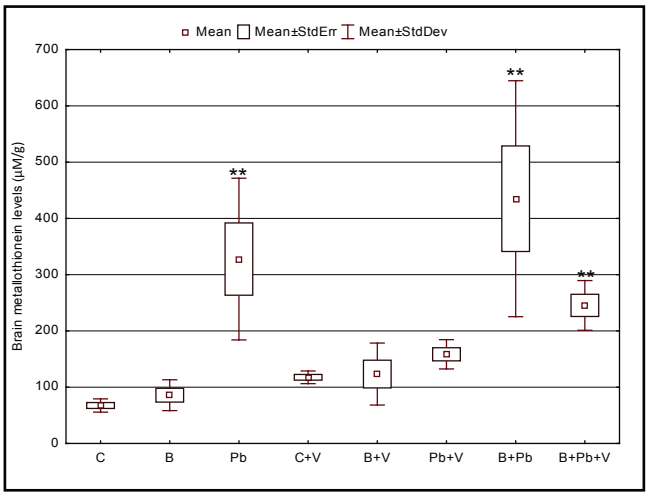

Fig. 3. Brain metallothionein levels in Japanese quail groups on day 30 of exposure. Groups C = control, B = exposure to cyanobacterial biomass, Pb = exposure to lead, C+V = vaccinated control, B+V = exposure to cyanobacterial biomass and Newcastle vaccination, Pb+V = exposure to lead and Newcastle vaccination, B+Pb = exposure to cyanobacterial biomass and lead, B+Pb+V = exposure to cyanobacterial biomass, lead and Newcastle vaccination. Measured in fresh weight tissue, n=5 in all groups, $^{**}p<0.01$.

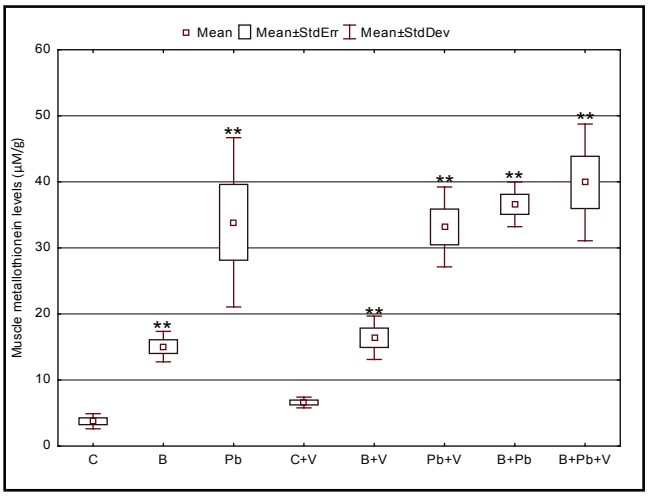

Fig. 4. Muscle metallothionein levels in Japanese quail groups on day 30 of exposure. Groups C = control, B = exposure to cyanobacterial biomass, Pb = exposure to lead, C+V = vaccinated control, B+V = exposure to cyanobacterial biomass and Newcastle vaccination, Pb+V = exposure to lead and Newcastle vaccination, B+Pb = exposure to cyanobacterial biomass and lead, B+Pb+V = exposure to cyanobacterial biomass, lead and Newcastle vaccination. Measured in fresh weight tissue, n=5 in all groups, $^{**}p<0.01$.

B+Pb and B+Pb+V (Figure 2). Brain MTs increased in all the Pb-exposed groups of birds, except for the group Pb+V (Figure 3). The highest increase in MTs levels in the pectoral muscle was found in the triple exposed group (Figure 4).

DISCUSSION

Standard ecotoxicological studies evaluate a range of exposure doses or concentrations to single substances and measure specific endpoints. To restrict the number of possible combinations, we examined one dose per each stressor to only account for environmentally relevant exposures. This is a simplification of the situation; however, it is close to what can happen under natural conditions. We have already used this approach several times to obtain some interesting results (Pikula *et al.* 2010; Bandouchova *et al.* 2011; Paskova *et al.* 2011; Ondracek *et al.* 2012; Osickova *et al.* 2012; Osickova *et al.* 2014; Ondracek *et al.* 2015).

Our data suggests that the MTs response in the tissues of the avian model species is driven mainly by the dietary exposure to cyanobacterial biomass and/or lead. Measurements of antioxidative enzymatic defense parameters provided similar results (Paskova *et al.* 2011). When comparing the relative strength of stressors which induce a MTs response in the tissues studied, the cyanobacterial biomass and lead seemed to be similar with one exception, i.e. in the brain where MTs induction was dominated by exposure to lead, which is understandable regarding the function of the blood-brain barrier (Sundstrom *et al.* 1985). The Newcastle disease vaccine induced the least response.

As the experiment lasted 30 days the toxic effects changed from acute to chronic. This was previously documented by the development of lead toxicity in blood collected in the course of the experiment. The most severe toxicity was observed on day 10 of the experiment, while hematocrit and hemoglobin levels had nearly normalised by day 30 (Pikula *et al.* 2010). Likewise, lead was probably sequestered from the blood and tissues to bones showing less toxicity (Pikula *et al.* 2013). Therefore, we might expect higher levels of MTs earlier in the course of the experiment. However, the design of our experiment only allowed the measurement of tissue MTs as a static value on day 30 of toxic exposure when birds were euthanised.

As expected, the concentration gradient of MTs in the tissues of the healthy control Japanese quails was similar to other animals (Pikula *et al.* 2010). Importantly, our study documents the efficient induction of MTs by multiple toxic stressors in birds (cf. Figures 1 to 4). The pattern of MTs induction and distribution among tissues most probably follows the route of uptake, accumulation and severity of action in target organs (Berntssen *et al.* 2001). Following the toxic exposure in our experiment there was a manyfold increase of MTs showing the ability of specific avian tissues to respond to the adverse effects of different stressors. To conclude, this study is in line with the notion that MTs are a sensitive but non-specific indicator of the toxic exposure of the organism.

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