Biochemical responses of juvenile and adult Japanese quails to cyanobacterial biomass

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Submitted: 2009-07-15 Accepted: 2009-08-24 Published online: 2009-10-20

Key words: **cyanotoxins; avian toxicity; biochemistry; low-molecular-weight antioxidants; total antioxidant capacity; acetylcholinesterase activity.**

Neuroendocrinol Lett 2009; **30**(Suppl 1): 199–204 **PMID:**20027171 NEL300709A34

Abstract

OBJECTIVES: The aim of the present study was to evaluate differences between juvenile and adult Japanese quails in responses to the exposure to cyanobacterial biomass in the diet.
DESIGN: The OECD 205 Guideline on Avian Dietary Toxicity Test (1984) was employed in the experiment. A total of 75 freshly hatched chicks and 30 adults were exposed to cyanobacterial biomass for 15 days and blood sampled daily and on days 5, 10 and 15, respectively. Japanese quail chicks and adults received the same daily dose of approximately 224.4 ng microcystins per gram of body weight. Biochemical responses were compared against controls.
RESULTS: No Japanese quail chicks and adults died during the acute 15-day-cyanobacterial-biomass exposure. Biochemical responses to the biomass in diet were first observed from day 5 post exposure to cyanobacterial biomass both in chicks and adults and there were age-related differences in the parameters changed. The responses of adult birds included an increase in lactate dehydrogenase, a drop in glucose and the total antioxidant capacity as well as a 15 to 20 % inhibition of acetylcholinesterase activity. Japanese quail chicks exposed to cyanobacterial biomass for the first 15 days after hatching reacted by having hypoproteinaemia, increased concentrations of triglycerides, uric acid and the total antioxidant capacity and a drop in high-density lipoprotein cholesterol in the blood.
CONCLUSIONS: Chicks were not found to be more susceptible to the effects of biomass exposure. It seems that, due to their physiological preparation for the oxidative stress associated with hatching, Japanese quail chicks were even better able to cope with the cyanobacterial-biomass-induced oxidative stress than adults.

ORIGINAL ARTICLE

Abbreviations & units

GSH – glutathione
GST – glutathione-S-transferase
HDL-C – high-density lipoprotein cholesterol
HPLC-DAD – high performance liquid chromatography with diode array detection
LMWAs – low-molecular-weight antioxidants
MC – microcystin
OECD – Organisation for Economic Co-operation and Development
TG – triglycerides
TP – total proteins
UAC – uric acid

INTRODUCTION

Over the last few decades there has been a growing body of reports on avian deaths around lakes and rivers co-occurring with cyanobacterial blooms in freshwater ecosystems (Alonso-Andicoberry *et al.* 2002; Chittick *et al.* 2002; Henriksen *et al.* 1997; Krienitz *et al.* 2002; Landsberg *et al.* 2007; Matsunaga *et al.* 1999; Murphy *et al.* 2000; Murphy *et al.* 2003; Onodera *et al.* 1997; Park *et al.* 2001; Wilde *et al.* 2005 and Wirsing *et al.* 1998). For example, Alonso-Andicoberry *et al.* (2002) described an event of catastrophic mortality in a Spanish national park during which about 60 % of greater flamingo chicks died.

Experimental studies on the effects of cyanobacterial toxins in birds include papers by Paskova *et al.* (2008), Skocovska *et al.* (2007) and Takahashi & Kaya (1993) using only adult males of the Japanese quail. While Takahashi & Kaya (1993) documented deaths between 14 and 18 hours after injection of a single dose of purified microcystin RR into the body cavity of Japanese quails and found the LD_{50} of MC-RR in quails in the dose of 256 μg/kg, Skocovska *et al.* (2007) reported no mortality during 10- and 30-day exposures to cyanobacterial biomass of known microcystins content. Oral exposure to environmentally relevant and higher doses of microcystins resulted in histopathological hepatic changes including cloudy swelling of hepatocytes, vacuolar dystrophy, steatosis and hyperplasia of lymphatic centres, increased activities of lactate dehydrogenase and a drop in blood glucose. Paskova *et al.* (2008) reported the induction of oxidative stress along with microcystins accumulation in birds exposed to natural cyanobacterial biomass. The effect of cyanobacterial exposure on the activation (P450-dependent 7-ethoxyresorufin-O-deethylase activity) and conjugation (GST, GSH) phase of detoxification metabolism, antioxidant activities and lipid peroxidation as a measure of oxidative damage in the exposed birds was investigated.

Endogenous antioxidant defences of enzymatic and non-enzymatic nature are critical for the control of reactive-molecular-species-mediated oxidative damage of biomolecules (Halliwell & Gutterdige 2007). It is known that low-molecular-weight antioxidants (LMWAs) such as ascorbic acid, uric acid, vitamin E, carotenoids, thiol-bearing substances and melatonin contribute to the protection of the organism against oxidative stress (Klandorf *et al.* 2001, Reiter *et al.* 2008, Surai 1999). Cells are equipped with defence mechanisms that provide protection via enzymatic activities or through LMWAs acting as chemical scavengers and neutralizing reactive molecular species (Chevion *et al.* 2000). LMWAs penetrate specific locations in the cell (Psotova et. al 2001), where oxidative stress may occur, and protect against reactive molecular species. It is possible to measure the total antioxidant capacity as a clinical marker of oxidative stress (Psotova *et al.* 2001).

Cholinesterase inhibitors such as organophosphorus and carbamate insecticides were recognized to cause acute intoxications in wild birds (Mineau & Whiteside, 2006). Most importantly, some cyanobacteria produce anatoxin-a mimicking acetylcholine and anatoxin-a(s) binding to acetylcholinesterase and preventing acetylcholine hydrolysis (Henriksen *et al.* 1997; Onodera *et al.* 1997; Krienitz *et al.* 2002). Moreover, repeated exposure to sub-lethal doses of acetylcholinesterase inhibitors may probably modulate the cholinergic anti-inflammatory pathway and alter the organism's innate immune system mediating responses to infection, injury and endotoxemia (Bernik *et al.* 2002).

Events of juvenile avian deaths are rather anecdotal. It was, therefore, the aim of the present study to test the hypothesis of freshly hatched chicks being more susceptible to the action of cyanotoxins than adults. For these purposes we evaluated differences in biochemical responses, the total antioxidant capacity derived from the low-molecular-weight antioxidants and acetylcholinesterase activities using a standard avian toxicity species. It may also be hypothesized that toxin-induced changes would be more pronounced in juvenile birds.

MATERIAL AND METHODS

Experimental design. Effects of cyanobacterial biomass in birds were evaluated according to the OECD 205 Guideline for the testing of chemicals—Avian Dietary Toxicity Test (1984) with some minor modifications to fit our experimental conditions.

The present study was started with one-day-old chicks and 4-month-old adults of the Japanese quail (*Coturnix coturnix japonica*). A total of 150 chicks, taken from the hatching device, were kept in two separate circles for controls and exposed birds with wood shavings used as bedding. Chicks were used without sexing as it is difficult to distinguish gender in one-day-old chicks as well as impractical because gender differences occur later in adults taking part in reproduction. Chicks were supplied with an artificial hen providing warmth, a commercial diet and water *ad libitum*. Following one day of acclimation experimental birds were given the cyanobacterial biomass using a crop probe once a day until day 15 of exposure (i.e. day 16 of age). ***Table 1*** reveals days of exposure, age of birds, their body weight and administered volumes of cyanobacterial

Table 1. Characteristics of Japanese quail chicks and the daily cyanobacterial biomass used in the experiment.

Exposure day	Age of birds (days)	Body weight (g)	Cyanobacterial-biomass volume administered (ml)	MCs sum in the daily dose (μg)
1–3	2–4	9–15	0.1	1.79
4–6	5–7	18–28	0.2	5.61
7–9	8–10	33–44	0.5	6.73
10–12	11–13	50–61	0.5	10.10
13–15	14–16	67–81	1.0	13.46

biomass together with the content of microcystins in the daily dose. Control birds were given drinking water in the same way. Five exposed and five control birds were selected on a random basis each day to be blood sampled by cardiac puncture using a heparinized set Omnican® 40 (Braun, Germany).

In total, 40 adult male birds were randomly divided into four groups of exposure for 5, 10, 15 days and controls and then they were kept individually. Males only were used to eliminate gender differences associated with metabolic changes of egg production. After one week of acclimation they were exposed to cyanobacterial biomass mixed with the feed (with the exception of controls). Water was supplied *ad libitum*. Adult birds were blood sampled from the jugular vein at days 5, 10 and 15 of exposure. Controls were sampled together with the 15-day-exposure group.

The weights of Japanese quails were daily determined using the AND GX-400 scales (A&D Instruments Ltd., Japan).

Experiments were performed in compliance with 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.

Experimental substances. The cyanobacterial biomass used in the present study was collected using a plankton net (25 μm) from the Musovska reservoir in August 2007. The biomass was dominated by *Microcystis* sp. including *M. aeruginosa* 90% and *M. ichtyoblabe* 10%. It was lyophilized and mixed into the dry diet for adults or used for the preparation of the liquid cyanobacterial biomass administered to chicks using a crop probe. Homogeneous distribution and stability of microcystins (MCs) in the diet and biomass was verified by laboratory analysis. The daily feed consumption was approximately 20 g per adult bird. In correspondence with Skocovska *et al.* (2007) the high exposure daily dose (i.e., the sum of MCs of 46 μg contained in 20 g of the diet) was selected for the experiment. Microcystin structural variants in the daily diet for adult birds thus included 19.84 μg MC-RR, 5.70 μg MC-YR and 20.46 μg MC-LR. The daily dose of microcystins per gram of body weight was approximately the same in Japanese quail chicks and adults (cf. Table 1). Microcystins content in biomass, the matter obtained by lyophilization and feed were analyzed using HPLC-DAD (Agilent 1100 Series) on Supelcosil ABZ+ Plus column, 150 x 4.6 mm, 5 μm according to Babica *et al.* (2006).

Biochemistry. Samples of blood were centrifuged immediately after collection, and plasma removed and frozen (-20 °C). Within a few days, plasma was analysed using an automated analyzer (SPOTCHEM™ EZ SP-4430, ARKRAY, Japan) for creatinine (μmol/l), aspartate aminotransferase (μkat/l), alkaline phosphatase (μkat/l), lactate dehydrogenase (μkat/l), creatine kinase (μkat/l), total protein (g/l), total cholesterol (mmol/l), high-density lipoprotein cholesterol (mmol/l), triglycerides (mmol/l), glucose (mmol/l), amylase (μkat/l) and uric acid (mmol/l).

Total antioxidant capacity. Cyclic voltammetry was used for estimation of the total antioxidant capacity derived from the plasma low-molecular-weight antioxidants (LMWAs). The measured anodic current is proportional to the concentration of LMWAs in the plasma sample. The principle of the assay employed in this study is described elsewhere (Chevion *et al.* 2000, Psotova *et al.* 2001). Measurements were performed using the EmStat device (PalmSens, Houten, Netherlands) equipped with platinum working (1 mm diameter), platinum auxiliary and silver/silver chloride electrodes screen-printed on a ceramic support (PalmSens). Electrodes were overlaid with 20 μl of plasma, and voltammetric curves were measured with a scanning rate of 100 mV/s. Data processing and device control were realized by the PSLite 1.7.3 software (PalmSens, Houten, Netherlands). The antioxidant capacity was estimated by means of the anodic current according to references (Zielinska *et al.* 2008, Psotova *et al.* 2001). Prior to measurements of experimental plasma samples the sensitivity of the device was tested and the cyclic voltammetry was calibrated by means of spiking plasma with ascorbate and cysteine. Cysteine was used as a representative of free thiol-bearing molecules that are oxidizable to the dithio-form. Ascorbate was selected as another molecule participating as an antioxidant in the body.

Assay of acetylcholinesterase activity in blood. Fresh blood (200 μl) was lyzed by mixing with 0.02 M TRIS HCl buffer (1.8 ml) with 0.01% Triton X-100. The lyzate was processed immediately due to the poor long term stability of acetylcholinesterase. Standard poly-

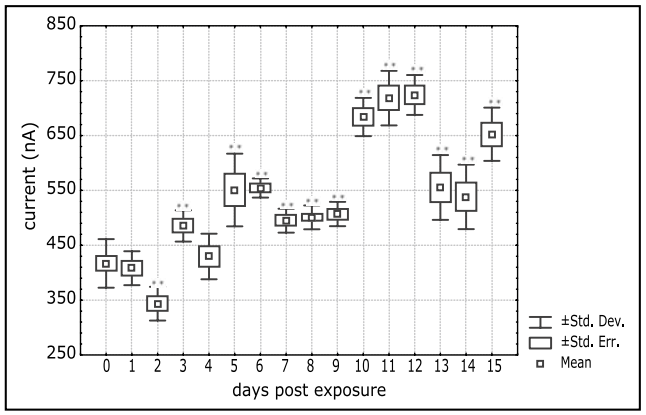

Figure 1. Total antioxidant capacity (low-molecular-weight antioxidants) in Japanese quail chicks receiving a daily dose of cyanobacterial biomass for 1 to 15 days in the diet, n = 5 in each group, ** = p<0.01 when compared against the control group. Oxidation current at 0.6 V obtained by performance of the voltammetric device was used as a measure of the total antioxidant capacity derived from low-molecular-weight antioxidants. Group 0 represents the range of values obtained when measuring control chicks throughout days 1 to 15 of the experiment (n=75).

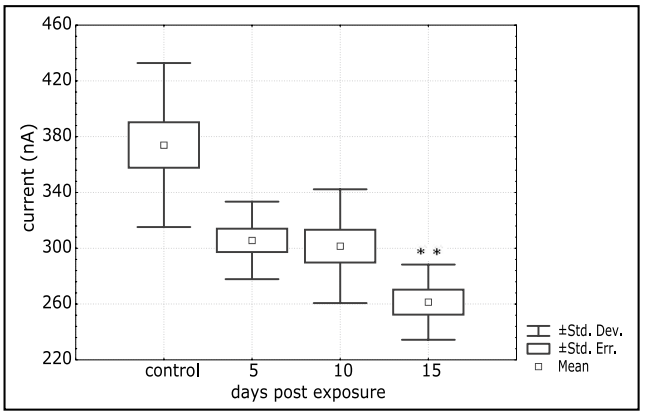

Figure 2. Total antioxidant capacity (low-molecular-weight antioxidants) in adult Japanese quails receiving a daily dose of cyanobacterial biomass for 5, 10 and 15 days in the diet, n = 10 in each group, ** = p<0.01 when compared against control group sampled on day 15 (n=10).

styrene cuvette was filled with Ellman's reagent [5,5´-dithiobis(2-nitrobenzoic acid)] (DTNB) - 4 mg/ml, 0.4 ml and 1.3 ml of previously prepared lyzate. The reaction started by the addition of 0.2 ml 1mM acetylthiocholine chloride. Absorbance was measured against blank (mixture of DTBN, TRIS-HCl, Triton X-100 and hemolyzate) at 412 nm after one minute incubation. The assay of acetylcholinesterase activity is described in greater detail elsewhere (Soukup *et al.* 2008).

Statistical analysis. Statistical analyses were performed with Statistica for Windows 7.0 (StatSoft, Tulsa, OK, USA). Data normality and the homogeneity of variances were evaluated by the Kolmogorov–Smirnov test and the Levene's test, respectively. One-way analysis of variance (ANOVA) and the nonparametric Kruskal–Wallis test were used for statistical comparisons. In the case of non-normal data distribution, nonparametric statistical analysis also included the Mann-Whitney *U* test. Values of $p<0.05$ and $p<0.01$ were considered statistically significant and highly significant, respectively, for all tests. Spearman rank order correlation analysis was employed to examine the relationship between the total antioxidant capacity (LMWAs) and plasma chemistry profiles.

RESULTS

No Japanese quail chicks died during the cyanobacterial-biomass exposure lasting 1 to 15 days. All adult birds exposed for 5, 10 and 15 days also stayed alive. Both the control and exposed chicks and adults did not show any clinical signs of disease or intoxication. There were also no differences in the development profile of body weights in groups of chicks and adults measured daily.

Considering the development profile of plasma chemistry parameters in controls and cyanobacterial-biomass-exposed Japanese quail chicks, no significant differences were found in glucose, creatinine, total cholesterol, amylase, creatine kinase, alkaline phosphatase, aspartate aminotransferase and lactate dehydrogenase. Uric acid levels were increased in the exposed chicks on days 5, 11 and 15 of exposure. The two-fold increase on day 5 was of high statistical significance ($UAC_{CONTROL\ 5}$=255±79 mmol/l; $UAC_{EXPOSED\ 5}$=532±103 mmol/l; p<0.01). The content of total proteins, compared daily, decreased from day 5 to day 15 of exposure, but only differences on days 5 and 14 were significant ($TP_{CONTROL\ 5}$=24.8±3.5 g/l, $TP_{EXPOSED\ 5}$=20.0±2.2 g/l; $TP_{CONTROL\ 14}$=26.6±3.7 g/l, $TP_{EXPOSED\ 14}$=22.0±2.0 g/l; p<0.05). On day 5 there was an increase in concentration of triglycerides ($TG_{CONTROL\ 5}$=1.5±0.5 mmol/l, $TP_{EXPOSED\ 5}$=2.20±0.2 mmol/l; p<0.05) and a decrease in high-density lipoprotein cholesterol levels of the exposed chicks compared to controls ($HDL\text{-}C_{CONTROL\ 5}$=4.7±0.8 mmol/l, $TP_{EXPOSED\ 5}$=2.8±0.4 mmol/l; p<0.01). Adult birds exposed to cyanobacterial biomass showed a two- to three-fold increase in lactate dehydrogenase activity ($LD_{CONTROL}$=4.6±2.3, $LD_{EXPOSED\ 5}$=9.6±1.2, $LD_{EXPOSED\ 10}$=12.3±1.5, $LD_{EXPOSED\ 15}$=15.6±2.4, p<0.01) and a drop in blood glucose ($GLU_{CONTROL}$=17.3±1.5, $GLU_{EXPOSED\ 5}$=9.9±1.1, $GLU_{EXPOSED\ 10}$=9.3±0.8, $GLU_{EXPOSED\ 15}$=10.6±2.7, p<0.05) on days 5, 10 and 15 (n=10 in each group) compared to controls. All other biochemical parameters remained unchanged in adults.

Figures 1 and 2 demonstrate differences in the development profile of the total antioxidant capacity derived from low-molecular-weight antioxidants in juvenile and adult Japanese quails. While after an initial drop in the total antioxidant capacity on day 2 there was a highly statistically significant increase on days 3 and 5 to 15 of exposure in Japanese quail chicks (cf. Figure 1), it was decreasing in adult birds during exposure (Figure 2). Figure 1 also demonstrates that the range of

values obtained when measuring the total antioxidant capacity in control chicks throughout days 1 to 15 of the experiment (Group 0) was relatively stable, i.e. not showing great variability and not differing significantly on individual days. Statistical analysis revealed significant correlations between uric acid levels and the total antioxidant capacity both in controls (n=75, R=0.57, p=0.001) and in cyanobacterial-biomass-exposed Japanese quail chicks (n=75, R=0.42, p=0.004).

Acetylcholinesterase activities in blood were evaluated only in adult birds exposed to cyanobacterial biomass for 5, 10 and 15 days and controls. Exposure for 10 and 15 days resulted in a decrease of about 15 to 20 %, respectively. It was not, however, statistically significant.

DISCUSSION

The hypothesis of greater susceptibility and more pronounced toxin-induced changes in freshly hatched chicks when compared to adults was tested using standard experimental avian species (Romijn *et al.* 1995). Naturally occurring cyanobacterial biomass collected from a lake in South Moravia in 2007 was used for this purpose. Japanese quail chicks and adults received approximately the same daily dose of 224.4 ng MCs per gram of body weight in the diet and, despite this, there were no mortality or clinical signs of toxicity observed. This finding corresponds with data reported by Skocovska *et al.* (2007).

Significant changes in biochemical responses were observed from day 5 post exposure to cyanobacterial biomass both in chicks and adults. However, there were age-related differences in the parameters changed. In correspondence with the study by Skocovska *et al.* (2007) the responses of adult birds included only changes in the activity of lactate dehydrogenase and glucose level. The elevation of lactate dehydrogenase may reflect hepatocellular damage due to various causes (Bandouchova *et al.* 2009, Ding & Ong 2003) and the drop in blood glucose may also be due to hepatopathy (Skocovska *et al.* 2007). Interestingly, these two parameters were not significantly changed in Japanese quail chicks exposed to cyanobacterial biomass. On the other hand, differences in biochemistry associated with the energetic metabolism included modified levels of triglycerides and high-density lipoprotein cholesterol in chicks. Hypoproteinaemia in chicks, not yet described in birds in association with exposure to cyanobacteria, may be caused by some resource allocation to counteract the effect of cyanotoxins as well as liver disease. Uric acid is a reliable indicator of kidney function (Bailey 2008). It is also considered one of the more important antioxidants in limiting the accumulation of glycosylated endproducts in birds and increased uric acid concentrations are lowering oxidative stress (Klandorf *et al.* 2001).

The induction of oxidative stress in cells and tissues exposed to cyanotoxins has been well documented (Ding & Ong 2003, Paskova *et al.* 2008). Age-related differences in the development of the total antioxidant capacity associated with cyanobacterial-biomass exposure are the most interesting findings of the present study. While the total antioxidant capacity derived from LMWAs had a decreasing trend with the ongoing cyanobacterial-biomass exposure in adult Japanese quails, it was increased considerably from day 5 to 15 after an initial drop in chicks on day 2 post exposure. These opposing trends may be explained by the development of antioxidant defence mechanisms in the chicken embryo to deal with oxidative stress at hatching time and to prepare for potential oxidative hazards after hatching. An accumulation of natural antioxidants, vitamins A, E and carotenoids, as well as an increase in glutathione peroxidase activity in the embryonic liver may have adaptive significance and were developed during evolution to protect unsaturated lipids from peroxidation during hatching stress conditions (Surai 1999). In light of the above facts and the lower degree of cyanobacterial-biomass-induced damage in juvenile birds it may be hypothesized that natural antioxidants provide a real protection against the effects of cyanotoxins in birds.

The advantage of cyclic voltammetry used in the present study is that it determines the total antioxidant capacity based on the evaluation of a total reduction effect of individual LMWAs without their exact qualitative differentiation (Psotova *et al.* 2001). Calibration of the measuring system was performed using ascorbate and cysteine. However, the number of chemical antioxidants occurring in the body is much more extensive (Salvi *et al.* 2002). The limit of detection of isolated compounds is in the range of 1–10 μM. This range of sensitivity is sufficient in determining physiological concentrations of biologically relevant scavengers. It has been found that ascorbic and uric acids greatly contribute to the measured antioxidant capacity (Kohen *et al.* 2000). In the present study uric acid levels correlated with the measured total antioxidant capacity. However, there was a closer correlation in controls than in exposed birds. Therefore, the increase in the total antioxidant capacity was not solely due to the greater uric acid concentration.

Interestingly, a 15 to 20 % inhibition of acetylcholinesterase activity in blood was found following exposure of adult birds to cyanobacterial biomass for 10 and 15 days, respectively. In general, a reduction in activity to lower than 20 to 50 % of normal values results in clinical signs and mortality (Poppenga 2007). Anatoxin–a(s) anticholinesterase activities and avian mortality cases were typically associated with *Anabaena flos-aquae* and *A. lemmermannii* (Henriksen *et al.* 1997, Onodera *et al.* 1997). The species composition of the cyanobacterial biomass used in the present study, however, included only *Microcystis* species. Despite the fact that the inhibi-

tion was insignificant from the statistical point of view, this finding should be further investigated.

Conclusion

It may be concluded that there were differences in biochemical responses to the exposure to cyanobacterial biomass between freshly hatched Japanese quail chicks and adults. However, chicks were not found to be more susceptible to the effects of biomass exposure. It seems that, due to their physiological preparation for the oxidative stress associated with hatching, Japanese quail chicks were even better able to cope with the cyanobacterial-biomass-induced oxidative stress than adults.

Acknowledgements

The present study was supported by the Internal Grant Agency of the University of Veterinary and Pharmaceutical Sciences Brno (Grant No. 121/2008/FVHE) and the Ministry of Education, Youth and Sports of the Czech Republic (Project MSM 6215712402 and 1M0571 Research Centre for Bioindication and Revitalization).

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