Effects of cyanobacterial biomass on avian reproduction: a Japanese quail model

Veronika Damkova [1], Jana Sedlackova [1], Hana Bandouchova [1], Lucie Peckova [1], Frantisek Vitula [1], Klara Hilscherova [2], Veronika Paskova [2], Jiri Kohoutek [2], Miroslav Pohanka [3], Jiri Pikula [1]

[1] Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic
[2] Centre for Cyanobacteria & Institute of Botany, Masaryk University Brno & Czech Academy of Sciences, Czech Republic
[3] Faculty of Military Health Sciences, University of Defence, Czech Republic

Correspondence to: Prof Jiří Pikula, University of Veterinary and Pharmaceutical Sciences Brno, Palackého 1-3, 612 42 Brno, Czech Republic
PHONE: +420-541 562 655, FAX: +420-541 562 657, E-MAIL: pikulaj@vfu.cz

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Abstract

OBJECTIVES: The present study was aimed at evaluation of the response of Japanese quails to cyanobacterial biomass administered in feed using biochemical profiles and parameters of reproduction.
DESIGN: Effects of cyanobacterial biomass were studied according to the OECD 206 Guideline on Avian Reproduction Toxicity. A total of 16 control and 16 experimental pairs (32 males and 32 females) were analyzed. The chronic exposure of parent birds lasted eight weeks with the daily sum of 61.62 μg MCs including 26.54 μg MC-RR, 7.62 μg MC-YR and 27.39 μg MC-LR.
RESULTS: There was no mortality both in control and cyanobacterial-biomass-exposed adults during the present study. Nor did the birds show any clinical signs of intoxication. Lactate dehydrogenase activity was increased about three-fold in exposed birds. No other biochemical parameters were showing significant differences. A total of 824 and 821 eggs were laid by control and exposed birds, respectively, during the eight-week study period. Eggs laid by cyanobacterial-biomass-exposed hens had lower weight than in controls (11.99±1.13g and 12.40±1.27g, respectively; $p<0.01$). Egg viability, hatchability, and the effect of hatching in control and experimental birds were 79.6±9.3 and 86.8±8.2% ($p<0.05$), 83.2±12.6 and 90.1±9.3%, and 65.2±17.7 and 77.7±15.2% ($p<0.05$), respectively. There was also a statistically significant difference in the number of 14-day old survivors per hen per day in control and experimental birds (0.38±0.02 and 0.43±0.01 %, respectively).
CONCLUSION: The lower weight of eggs produced by exposed parental hens was not reflected in their biological quality. On the contrary, reproductive parameters in cyanobacterial-biomass-exposed birds were better than in the control group. It might be hypothesized that compounds of hormonal activity could be present in the complex cyanobacterial biomass. However, further research into this issue is necessary.

ORIGINAL ARTICLE

Abbreviations & units

HPLC-DAD – High performance liquid chromatography with diode array detection
MC – Microcystin
OECD – Organisation for Economic Co-operation and Development

INTRODUCTION

Wild birds may be exposed to a great variety of potential toxicants (Poppenga 2007). Origins of avian toxicology are in the recognition of the effects of organochlorine insecticides leading to rapid declines of many bird populations (Carson 1962). Wild birds are very conspicuous in the landscape and injuries to populations of birds from environmental pollutants and pesticides are obvious indicators of environmental damage (Fry 1995). Birds, like other groups of animals (Blahova *et al.* 2008) and humans (Kruzikova *et al.* 2008), may be subject to mortality or adverse biological effects due to the action of both natural toxins and man-made pesticides and industrial chemicals.

One important form of pollution is nutrient pollution, especially with phosphorus but also with nitrogen, leading to the trophication of aquatic ecosystems and, consequently, to the accelerated growth of algae and cyanobacteria as well as higher forms of plant life capable of upsetting the balance of organisms present in the water and impairing the quality of the water concerned. Cyanobacterial proliferations, also known as blooms, can have a major impact on ecosystem functioning and on the health of animals and humans (Briand *et al.* 2003). The most serious consequences of bloom formation are poisonings by secondary metabolites of cyanobacteria - cyanotoxins (Carmichael 1992), which fall into five groups (Wiegand and Pflugmacher 2005) based on their primary toxicological target.

Cyanobacterial products have also been recognized to play a role in avian wildlife mortality (Alonso-Andicoberry *et al.* 2002, Chittick *et al.* 2002, Henriksen *et al.* 1997, Krienitz *et al.* 2002, Matsunaga *et al.* 1999 and Onodera *et al.* 1997). The toxicity of cyanobacteria in wild birds has been suggested rather from indirect evidence such as the observation of mortality in several avian species under natural conditions associated with the occurrence of water blooms and long-term stay and foraging of birds at the affected locality, the detection of cyanobacterial toxins in the crop and liver and the exclusion of alternative causes of mortality. There are two papers complementing field observations with laboratory experiments of evidence-based avian toxicity tests using a standard experimental avian species and evaluating the effects of different doses of cyanobacterial toxins in birds (Paskova *et al.* 2008, Skocovska *et al.* 2007). These studies showed, however, that daily ingestion of environmentally relevant doses of cyanobacterial biomass containing microcystin for 10 and 30 days resulted in no mortality. Under natural conditions, birds may certainly be exposed to cyanobacterial toxins for a longer period than 10 and 30 days in the above-mentioned acute and subchronic experiments. However, there are no studies focused on the effects of chronic exposure of birds to cyanotoxins.

It was, therefore, our goal to investigate the effects of sub-lethal chronic exposure to cyanobacterial biomass of known microcystins content in order to obtain data on health, biochemical as well as reproductive responses of model bird species.

MATERIAL AND METHODS

Experimental design. The effects of cyanobacterial biomass in birds were evaluated according to the OECD 206 Guideline for the testing of chemicals—Avian Reproduction Toxicity Test (1984) with some minor modifications to fit our experimental conditions.

Briefly, a total of 80 healthy Japanese quails (2 months old *Coturnix coturnix japonica*) from the same hatch were randomly divided into 40 breeding pairs and placed into cages appropriate for this avian species for three weeks of acclimation. The onset of egg laying was stimulated by increasing the photoperiod to 16 hours a day and supplying the birds with a feeding mixture for laying hens plus a mineral supplement NutriMix (Biofaktory, Prague, Czech Republic) and Hydrovit E+Se (PharmaGal, Nitra, Slovak Republic). A two-week pre-treatment period was started as the egg production was peaking. Again on a random basis, 20 control and 20 experimental pairs were allocated into the control and exposure group. Birds were weighed and blood sampled at this time. The pre-treatment period made it possible to select the final 16 control and 16 exposed pairs meeting the validity criteria of the test. The parental cages were distributed in such a way that positional effects were avoided. During the treatment period, the experimental pairs were exposed to cyanobacterial biomass mixed with the feed for eight weeks. The control Japanese quail pairs were fed the same diet without the cyanobacterial biomass. At the end of the eight-week-treatment period all adult birds were blood sampled, euthanized and necropsied. The experiment continued for another four weeks during which the last batches of chicks hatched and were kept for 14 days in order to observe any clinical signs and perform their body weight measurements.

During the pre-treatment and treatment period, all eggs, except for those that were cracked, broken or abnormal or used for eggshell measurements, were artificially incubated and allowed to hatch. All offspring were maintained on an untreated diet until 14 days after hatching. Eggs were collected twice a day, numbered according to the cage of origin and stored; broken eggs were numbered and recorded. The eggs were stored in a cold storage facility at 13 °C and 55 – 75 % relative humidity, for up to six days, prior to setting in the incubator. Eggs were set in an incubator with temperature and humidity control and an automatic turn-

ing device (BIOSKA Sedlčany, Czech Republic) once per week. Fertility and embryo viability were checked by candling the eggs on day 8 of incubation. All eggs that appeared to contain live embryos were placed back into the incubator. Eggs that did not appear to contain a live embryo were opened up and examined under a dissecting microscope in order to distinguish between infertility and early embryonic death. Fertility, infertility, viability and embryonic death were recorded. On day 15 of incubation, the eggs were candled again for viability of embryos; all live embryos were transferred to a hatcher (BIOSKA Sedlčany, Czech Republic) and recorded as late viable embryos. Embryos identified as dead after day 15 were recorded as late embryonic death. One seventh of the egg production was not incubated but used for eggshell thickness and microcystin content measurements.

The 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 diet. Homogeneous distribution and stability of microcystins (MCs) in the diet was verified by laboratory analysis. The daily feed consumption was estimated as 20 g per bird. In correspondence with Skocovska *et al.* (2007) the high exposure daily dose (i.e., sum of 46 μg MCs contained in 20 g of the diet) was selected for the experiment. Microcystin structural variants in the daily diet included 19.84 μg MC-RR, 5.70 μg MC-YR and 20.46 μg MC-LR. 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 × 4.6 mm, 5 μm according to Babica *et al.* (2006).

Data collection. Data collected during the study included the mortality of adults, their feed consumption, body weights, clinical observations, any pathological findings and organ weights (liver, spleen, testes, ovary, oviductus). Reproductive parameters included the number of eggs laid, eggs cracked and broken, egg abnormalities, eggs set, eggshell thickness, eggs fertile, embryos viable, normal or abnormal hatchlings, clinical signs of toxicity, abnormalities and mortality, 14-day-old surviving chicks, chick body weight at hatching and 14 days after hatching. Reproductive indices such as viability (percentage of fertile eggs set), hatchability (percentage of chicks hatching from fertile eggs) and the effect of hatching (the percentage of chicks hatching from all eggs set), the mean number of 14-day old survivors per hen per day and the number of eggs laid per hen per day were calculated.

Weights of parental birds, eggs, chicks, organs at necropsy and feed consumption were determined using the AND GX-400 scales (A&D Instruments Ltd., Japan).

Biochemistry. Samples of blood for plasma chemistry profiles were collected from all birds in the experiment at the end of the acclimation period as well as at the time of termination of adults. Blood (1 ml) was collected from the right jugular vein using the Omnican® 40 (Braun, Germany). Whole blood was placed in heparinized tubes (Leciva inj., Prague), centrifuged immediately, 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).

Statistical analysis. Statistical analyses were performed with Statistica for Windowss 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.

RESULTS

There was no mortality in both control and cyanobacterial-biomass-exposed adults during the present study. Nor did the birds show any clinical signs of intoxication. The mean daily feed consumption was 26.79 g per experimental bird. It means that parental birds were exposed to the daily sum of 61.62 μg MCs including 26.54 μg MC-RR, 7.62 μg MC-YR and 27.39 μg MC-LR. Feed consumption of control and experimental birds did not differ statistically. No differences were found between the body weights of parental adults from the control and exposed groups, both at the start of the pretreatment period and then at weekly intervals during the study.

Biochemical analysis revealed a three-fold increase of lactate dehydrogenase activity in exposed parental birds compared to controls (n=32 in each group, $p<0.01$, ***Figure 1***). Differences in other examined biochemical parameters were not statistically significant. Organ weight comparisons of exposed and control adults resulted in finding 1.17 times heavier spleens after two months of exposure to cyanobacterial biomass in males

(n= 16 in each group, p<0.05). However, spleen weights were about the same in control and exposed females.

A total of 824 and 821 eggs were laid by control and exposed birds, respectively, during the eight-week study period and the number of eggs laid per hen per day equalled 0.91 in both groups. Eggs laid by cyanobacterial-biomass-exposed hens were lower in weight when compared to controls with high statistical significance (cf. ***Figure 2***). The same is true for the body mass of chicks at hatching and 14 days after hatching. No differences were found in the eggshell thickness. Both the early and late embryonic death rates were lower in eggs from exposed birds than controls, but without statistical significance. No differences were also found in the 14-day survival of chicks and the chicks showed no clinical signs of intoxication or any abnormalities.

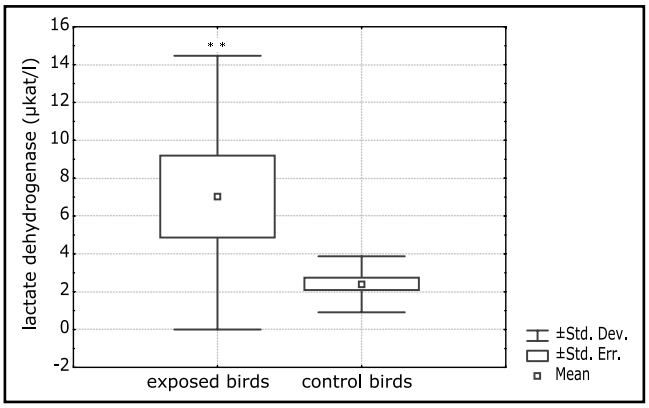

Figure 1. Comparison of LDH activities of exposed and control Japanese quails. Number of animals (16 pairs in both groups), ** =p<0.01.

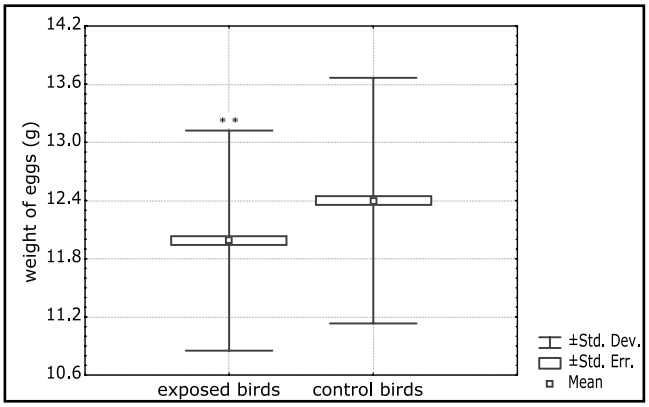

Figure 2. Comparison of weights of eggs laid by exposed and control Japanese quail hens (n = 821 and 824, respectively; ** =p<0.01).

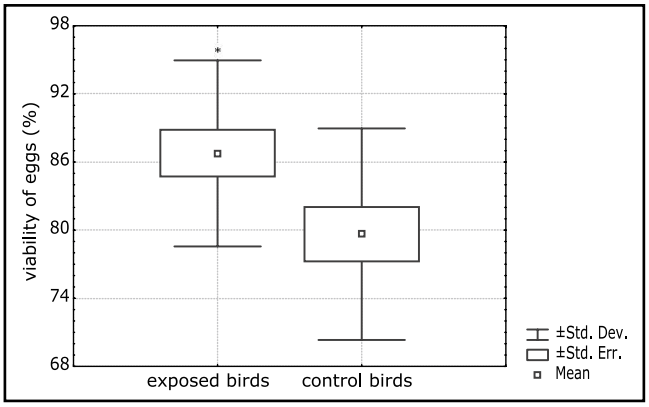

Figure 3. Viability of eggs laid by exposed and control Japanese quail hens (n = 821 and 824, respectively; * =p<0.05).

Egg viability rates in control and exposed birds were 79.6±9.3 and 86.8±8.2 %, respectively (cf. ***Figure 3***). Hatchability rates in control and cyanobacterial-biomass-exposed birds reached values of 83.2±12.6 and 90.1±9.3 %, while the overall effect of hatching values were 65.2±17.7 and 77.7±15.2 %, respectively. Statistically significant differences (p<0.05) were also found in fertilization rates (viability) and the overall effect of hatching. There was also a statistically significant difference (p<0.05) in the number of 14-day old survivors per hen per day in control and experimental birds (0.38±0.02 and 0.43±0.01 %, respectively).

DISCUSSION

In this experimental study, the response of Japanese quails to the chronic oral exposure to cyanobacterial biomass containing defined amounts of microcystins was evaluated using biochemical profiles of blood parameters and parameters of reproduction. To the best of our knowledge, this was the first experiment to study the long-term effects of cyanobacteria in birds providing evidence-based results because other papers report data on a single dose (Takahashi and Kaya 1993) or 10- and 30-day exposures only (Paskova *et al.* 2008, Skocovska *et al.* 2007). Interestingly, while biochemistry revealed some adverse effects of chronic exposure to cyanobacterial biomass in Japanese quails, the reproduction seemed to be rather stimulated.

Considering biochemistry, the only difference of statistical significance was the increase of lactate dehydrogenase activity to about a three-fold level in exposed birds. This finding is in agreement with previous results from acute and sub-chronic exposures (Skocovska *et al.* 2007). Leakage of lactate dehydrogenase from hepatocytes follows functional alterations of mitochondria and its elevation may reflect a low degree of hepatocellular damage due to various causes (Bandouchova *et al.* 2009, Ding and Ong 2003). Skocovska *et al.* (2007) also reported a significant drop in the blood glucose level in cyanobacterial-biomass-exposed birds. However, there were no differences in the blood glucose of experimental and control birds in the present study despite the fact that due to the higher-than-expected feed consumption, daily doses of microcystins amounted to about 130 % of those used previously (Skocovska *et al.* 2007).

Takahashi and Kaya (1993) reported death and spleen enlargement in Japanese quails following the intraperitoneal injection of microcystin RR with the LD_{50} of 256 µg/kg. The present exposure resulted in a significantly enlarged spleen in parental male birds and unchanged

in females. Skocovska *et al.* (2007) expressed the opinion that the death of birds following the intraperitoneal injection of microcystin, as reported by Takahashi and Kaya (1993), was rather due to its antigenic action demonstrated by the accumulation of lymphocytes in the spleen than toxicity. In light of the present finding, and knowing that Takahashi and Kaya (1993) used only males for their study, it may be stated that some other mechanisms, including the toxic ones, might be responsible. The gender difference in the spleen response is, however, hard to explain.

The adverse effects of chronic exposure to cyanobacterial biomass were also demonstrated by the significantly lower weights of eggs laid by treated hens and then of chicks at hatching and 14 days after hatching from these eggs when compared to controls. There was probably some resource allocation for counteracting the effects of the exposure that the exposed laying hens were then lacking in the egg production. Most importantly, however, the lower weight of eggs produced by exposed parental hens was not reflected in their biological quality. On the contrary, reproductive parameters in cyanobacterial-biomass-exposed birds were better than in the control group. It is not what was expected because vacuolar degeneration of the germinative epithelium in the testicular tissue was previously noticed (Skocovska *et al.* 2007).

The results demonstrating reproductive stimulation by cyanobacterial biomass in this avian species are rather surprising. However, the laboratory testing procedure employed (i.e., Avian Reproduction Toxicity Test OECD 206) is well suited to assess the effects of pesticides and other chemicals upon avian health and reproduction because integrated biological endpoints such as the number of 14-day old survivors per hen per day, egg production, fertility, embryonic mortality, hatchability and chick survivorship are evaluated to enhance both the statistical power and biological impact of the test to detect effects and provide information on the mechanisms of toxicity that may adversely affect the overall reproductive success. It is also necessary to stress the fact that all validity criteria of the test were met in this study.

The reproductive effects of cyanobacterial biomass in Japanese quails are to some extent similar to results described by Reinikainen *et al.* (1999) investigating experimentally how resources were allocated to reproduction in *Daphnia pulex* and *Daphnia longispina* when varying levels of toxic *Microcystis* were added to higher quality food. Water fleas produced small clutches but the presence of cyanobacteria increased the portion of available resources allocated to reproduction. The observed allocation may be a means of maximizing reproduction under diminished longevity.

Hormesis, a dose-response phenomenon characterized by low-dose stimulation and high-dose inhibition, might be another mechanism participating in the observed reproductive stimulation of quails due to the chronic exposure to cyanobacterial biomass (Calabrese 2008). Hormesis has been noted in numerous studies with over 50 chemicals and even in poultry species fed with low levels of aflatoxin (Diaz *et al.* 2008).

The ratio of about 24 g to 20 kg of the lyophilized cyanobacterial biomass and the feeding mixture for laying hens was low and could not be responsible for the so-called nutritional flushing effect in exposed birds because analyses of the nutritional value of the diets with and without the cyanobacterial biomass supplement showed no differences. Cyanobacteria contain a great number of compounds of potential biological activity with various mechanisms of action and some studies have documented significant biological effects independent of the known cyanotoxins content. Their impact on avian wildlife populations is largely not known (Landsberg *et al.* 2007). However, most authors expect negative effects. On the other hand, it cannot be ruled out that complex biomass might contain some biologically active compounds potentially stimulating reproduction, similarly to phytohormones in plants.

Conclusion

There was no mortality or any clinical signs of intoxication in cyanobacterial-biomass-exposed as well as in control birds during the present experiment. Interestingly, reproduction parameters in experimental birds were better than in the control group. We may hypothesize that, apart from microcystins, compounds of hormonal activity can be present in the complex cyanobacterial biomass. However, further research into this issue and the character of the compounds is necessary.

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