Vertical transmission of microcystins to Nile crocodile (*Crocodylus niloticus*) eggs

Alukhethi Singo[a], Jan G. Myburgh[a], Peter N. Laver[b], Elizabeth A. Venter[a], Gezina C.H. Ferreira[a], Gertruida M. Rösemann[c], Christo J. Botha[a,*]

[a]Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Republic of South Africa

[b]Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, Republic of South Africa

[c]National Horseracing Authority of Southern Africa, Johannesburg, Republic of South Africa

Corresponding author. Tel.: +27 125298023; fax +27 125298304

E-mail address: christo.botha@up.ac.za (C. J. Botha).

Abstract

Cyanobacteria or blue green algae are known for their extensive and highly visible blooms in eutrophic, stagnant freshwater bodies. Climate change and global warming have also contributed to a rise in toxic cyanobacterial blooms. One of the most important cyanobacteria is *Microcystis aeruginosa*, which can synthesize various microcystins that can affect the health of terrestrial and aquatic animals. Commercial Nile crocodile (*Crocodylus niloticus*) farming in South Africa is based on keeping breeders (adult males and females) in big dams on farms (captive-bred approach). Unfortunately, cyanobacterial blooms in the breeder dams are a concern to farm owners, managers and veterinarians. The main objectives of this research project were to determine if microcystins were present in the contents of crocodile

eggs and the liver and yolk of dead hatchlings, and to determine if the reduced hatchability on commercial farms might be caused by these toxins. Furthermore, the concentration of microcystins in the breeder dam was monitored on a monthly basis spanning the ovulation and egg laying period. During the hatching season microcystin concentrations in unfertilised eggs, egg shell membranes and in the yolk and liver of dead hatchlings were determined using liquid chromatography-high resolution mass spectrometry (LC-HRMS). Microcystins were detected in Nile crocodile egg and hatchling samples. Microcystin (MC-LR, MC-RR, MC-YR) concentrations in the crocodile egg and hatchling samples collected from clutches with a good hatching rate (≥ 90%) ranged between 0 - 1.76 ng g^{-1}, with the highest concentration in the eggshell membranes. Microcystin concentrations in samples collected from clutches with a bad hatching rate (≤ 10%) ranged from 0 – 1.63 ng g^{-1} with the highest concentration detected in the hatchling yolk. However, the concentrations were probably underestimated as the percentage recovery from spiked samples was very low with the extraction method employed. Bayesian analysis suggests that the liver, yolk and unfertilised egg all have similar microcystin concentrations, while the membranes have (with moderate to high certainty) higher microcystin concentrations. There appears to be no difference in microcystin concentrations among good and bad clutches across all tissue types or within a specific tissue type, but due to the small sample size, it was not possible to determine whether microcystin affected the hatchability of Nile crocodile eggs. However, vertical transmission of microcystin variants to the Nile crocodile egg does occur and the possible implications for the survival of wild Nile crocodile populations should be ascertained.

Keywords: Climate change, Cyanobacteria, Eggs, Microcystin, Nile crocodile, Vertical transmission

1. Introduction

Nile crocodile (*Crocodylus niloticus*) farming is of economic importance to South Africa as belly skins are exported for the manufacturing of high-end leather items such as handbags and shoes (Van As, 2016). In addition, the flesh of the Nile crocodile is internationally available on menus of *haute cuisine* restaurants (Flint et al., 2000). Trade in captive-bred Nile crocodile products is legal under the Convention on International Trade in Endangered Species (CITES) (Luxmoore, 1992). No harvesting of crocodile eggs from the wild is allowed in South Africa as wild Nile crocodile populations are declining (Ashton, 2010; Combrink et al., 2011; Lane et al., 2013). All commercial farm crocodile offspring come from breeding stock kept on commercial farms (captive-bred approach). Unfortunately, cyanobacterial blooms are frequently present in breeder dams on most commercial farms and are a concern to farm owners, managers and veterinarians (Fig. 1). Farmers implicate cyanobacterial blooms as a serious potential cause for the low hatching rate of crocodile eggs and survivorship of the hatchlings.

Fig. 1. A crocodile in the breeder dam covered with a layer of cyanobacteria from a bloom.

High temperatures in summer and an increased level of eutrophication favour cyanobacterial growth and multiplication (Reichwaldt and Ghadouani, 2012). Currently, climate change and global warming augment the increase in harmful cyanobacterial blooms in rivers or dams (Oberholster et al., 2009; Paerl and Huisman, 2009). The cyanobacteria release cyanotoxins, either hepato- or neurotoxins (Carmichael, 1992). *Microcystis aeruginosa* is arguably the most important bloom-forming organism that synthesizes microcystins in South Africa (Van Ginkel, 2003, Ndlela et al., 2016). Different variants of microcystins have been isolated such as microcystin-LR (MC-LR), MC-RR and MC-YR (Carmichael, 1992). Spoof and Catherine (2017) listed 248 variants of microcystin which have thus far been identified.

Various deleterious effects of microcystins on early developmental stages of different fish species have been reported (Malbrouck and Kestemont, 2006). Exposure of zebra fish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*) and chub (*Leuciscus cephalus*) eggs to 0.5, 5 and 50 µg L^{-1} MC-LR, MC-RR and MC-YR elicited no toxic effects during embryonic development (Oberemm et al., 1999). However, exposure to the two higher MC-LR and MC-RR concentrations resulted in a reduced survival rate of the zebra fish larvae and changed the time to hatching (either advanced or delayed) of the rainbow trout embryos (Oberemm et al., 1999). Microinjection of Japanese medaka (*Oryzias latipes*) embryos with 0.1 - 10 µg ml^{-1} MC-LR induced dose dependant mortality that reduced survival rates by up to 90% (Jacquet et al., 2004). Liu et al. (2002) also reported that freshwater loach (*Misguruns mizolepis*) embryos were more sensitive to the toxic effects of MC-LR when exposed during later developmental stages.

Chen and co-workers (2009) detected microcystins (MC-LR, MC-RR and MC-YR) in the gonads of a turtle species (Chinese softshell turtle; *Pelodiscus sinensis*), domestic duck (*Anas platyrhynchos*) and a water bird, the Black-crowned night heron (*Nycticorax nycticoraxs*), thereby suggesting the possible vertical transmission of microcystins during egg formation. Furthermore, microcystin was also present in the egg yolks and egg whites of the heron and duck and the authors proposed a potential embryotoxic effect in these species

(Chen et al., 2009). Severe lesions were observed in the ovaries of common bream (*Abramis brama*) collected from three lakes in the Ile-de-France region with seasonal or perennial cyanobacterial blooms and the authors surmised that there could be an effect on reproduction which can impact the fish population fitness and sustainability (Trinchet et al., 2013)

The main objective of this study was to determine if there is vertical transmission of microcystins to Nile crocodile eggs. To our knowledge there is no scientific report that documents microcystin concentrations in Nile crocodile eggs or in the tissues of hatchlings. As harmful blooms could also negatively impact the survival of wild Nile crocodile populations the study also investigated if the poor hatching rate and survivorship of the hatchlings recorded at a commercial crocodile farm could be attributed to the presence of microcystins in the eggs and hatchlings. In addition, microcystin concentrations in a crocodile breeding dam were determined over a nine-month period that included the breeding and egg-laying periods.

2. Materials and methods

2.1 Water samples

2.1.1 Collection

Water samples (5 L) were collected every month (August 2014 - September 2015) at the breeding dam of a commercial Nile crocodile farm in the North-West Province of South Africa. A bucket, attached to a rope, was lowered from the bridge spanning the crocodile breeding dam to collect a sub-surface water sample.

2.1.2 Cyanotoxin extraction

The water samples were frozen (-20°C) and thawed three times to break the cyanobacterial cells, contained in the water, in order to release intracellular cyanotoxins. After sonication

(Ultrasonic LC60, Elma, Germany) the samples were filtered through a nylon net (90-µm mesh size) to remove impurities. The cyanotoxins were extracted following a modified method as reported by Rundberget and co-workers (2009). Briefly, 1 L of the sample was added to 3 g of activated Diaion HP-20 resin (Sigma-Aldrich, Germany). The resin was activated by soaking once in 100% methanol for 15 min, followed by soaking three times in ultrapure water (Milli-Q^{50}, France) for 5 min periods, according to the manufacturer's (Supelco) instructions. The resin and samples were shaken overnight for 19 h on a linear shaker (Model 202, Labotec, South Africa) to allow sufficient contact time for the cyanotoxins to be adsorbed by the resin. The resin was then transferred to a 25 ml Varian Bond-elute reservoir plugged with non-absorbent cotton wool and washed free of salts using 30–50 ml ultrapure water. Excess water was removed from the column with the aid of a plunger. Ten ml methanol was added to the column and the resin was stirred gently, and left to stand for 15 min. The column was eluted slowly (0.5–1 drop s^{-1}) and when finished, the process was repeated with a further 10 ml methanol. Finally, an additional 3 ml methanol was used to flush the remaining cyanotoxins from the column. The eluted extract was kept in the freezer at -20°C in 20 ml screw cap glass tubes.

2.1.3 Enzyme linked immunosorbent assay (ELISA)

Some of the methanol samples were used for microcystin analysis using an ELISA (Abraxis ADDA ELISA kit). The assay was performed following the manufacturer's instructions. Analysis of the samples was conducted in duplicate. The methanol extracted samples were diluted to ensure that the concentration of the methanol did not exceed 5% to avoid false positive results. Absorbance was read at 450 nm using a microplate reader (Biotek Synergy GEN 5) within 15 min after the addition of the "stop solution" to quench the reaction. Abraxis ELISA data were analysed using the 4-parameter logistic fitting with Excel Solver for Microcystin ELISA Version 20060924 (ELISA Software) supplied with the Abraxis kit.

2.2 Crocodile egg and hatchling samples

2.2.1 Collection

At the commercial crocodile farm, courtship and mating occurs from July to August and the females lay clutches of eggs near the breeding dam in nests and cover them with dirt and sand during September and October. Eggs are collected every morning during the egg laying season, the clutches identified and all the eggs of a clutch are kept together and incubated at 31-32 °C until hatching. After an average incubation period of 76 days the eggs hatch. The crocodile farm's hatching season lasts from late November until mid-February. Sample collection was undertaken during the peak of the hatching season, December 2014 to January 2015. Samples were collected from so called 'good clutches' (≥90% hatching rate and survivorship) and 'bad clutches' (≤10% hatching rate and survivorship). Samples (n = 2-10) were collected from different 'good' and 'bad' clutches during the beginning ('early'), middle and towards the end ('late') of the hatching period. The number of samples available was influenced by the hatching rate of the eggs. The liver, yolk, unfertilised egg and egg membrane samples were transported on ice in a cooler box to the laboratory where the samples were immediately frozen at −20 °C. One control liver was collected from the farm's abattoir during routine slaughter.

2.2.2 Sample processing

The samples were thawed and divided into three groups for analysis based on the period collected during the season (early, middle and late collection); the samples of different clutches were pooled and homogenized in a glass beaker using a homogenizer (BIO-GEN PRO200, Pro Scientific, USA). A maximum of 10 samples were pooled and homogenized (Table 2).

2.3 Liquid chromatography–high resolution mass spectrometry (LC-HRMS)

2.3.1 Chemicals and equipment

HPLC-grade methanol, acetonitrile and hexane were obtained from Burdick and Jackson, USA. Ammonium acetate (>98%) and formic acid (98-100%) were obtained from Sigma-Aldrich Chemie, Germany. Clean-up C18 (6cc, 500mg) solid phase extraction (SPE) cartridges were obtained from UCT, USA. A GM200 Knife Mill was obtained from Retsch GmbH, Germany and an IKA Ultraturrex homogeniser was obtained from Zymark Turbovap.

2.3.2 Preparation of the calibration curve

Standards of MC-LR, MC-RR and MC-YR were purchased from Sigma. A combined standard was prepared in methanol containing 1000 ng ml^{-1} of each of the microcystins. Serial dilutions were made to obtain a range between 500 and 62.5 ng ml^{-1}. The liver sample of the slaughtered crocodile and chicken egg samples were spiked with MC-LR, MC-RR and MC-YR (Abraxis) and were analysed by LC-HRMS to determine the percentage recovery of the three microcystin variants.

2.3.3 Analysis of water samples

The methanol-extracted water samples were weighed to determine the exact volume and evaporated under nitrogen until dry. Samples were dissolved in 200 µl methanol. An aliquot of each sample (20 µl) was diluted to total volume of 520 µl and analysed. Samples that exceeded the calibration range were diluted further and analysed.

2.3.4 Extraction method for the tissue samples

Tissue samples were extracted according to Bruno and co-workers (2009). Quality control (QC) for each sample was prepared for each tissue type from the samples supplied. An aliquot of the milled samples and QCs were weighed and homogenized in 10 ml 5% methanol in acetonitrile. The samples were centrifuged, the supernatant transferred to a clean tube and the pellet re-extracted. The combined supernatant contained visible fat and a

10 ml hexane wash was introduced to remove excess fat. The supernatant was then evaporated under nitrogen. Dried samples were dissolved in 1 ml methanol, 4 ml water was added and it was applied to the SPE columns which were conditioned with 2 ml methanol followed by 2 ml water. Columns were washed with 5 ml methanol, followed by 5 ml 1% formic acid in methanol. Elutes were dried under nitrogen and reconstituted in 200 µl methanol and analysed.

2.3.5 High performance liquid chromatograph conditions

The high performance liquid chromatography (HPLC) was performed using an Agilent 1260 Infinity instrument (Agilent, California, USA) with an XSelect CSH C18 column 150 × 2.1 mm, 5 µm particle size (Waters Corporation, USA). The column temperature was 40°C and the flow rate 350 µl min^{-1}. The mobile phase was a gradient of water to acetonitrile, both containing 5 mM ammonium acetate and 1.0% formic acid. The linear gradient started at 2 min from 2% to 98% acetonitrile at 20 min, kept at 98% to 26 min, followed by a return to initial conditions at 28 min. The column was allowed to recondition until 35 min when the next injection started.

2.3.6 High resolution mass spectrometer conditions

Mass spectrometric analysis employed a Thermo Fisher QExactive HRMS (Thermo Fisher, USA) controlled by Xcalibur software. The scan range was from 100 to 1100 *m/z* units with a resolution of 140 000 in positive mode. The AGC target was set to $3x10^{-6}$ and the maximum injection time set to 200 msec. The instrument interface was an Ion Max API source fitted with a HESI-II probe. Tune file settings were: heater temperature at 400°C, capillary temperature at 250°C, the sheath gas flow rate at 50, the aux gas flow rate at 10 and sweep gas was switched off. The source voltage in positive ion mode was 3.5 kV and the S-lens was set at 55 V. Quantitative analyses were performed using Xcalibur Quanbrowser software.

2.4 Statistical analysis

Microcystin data approximately followed a lognormal distribution, thus data were log-transformed using *log*(x+0.01) where appropriate. Effect sizes and group means are reported as back-transformed values, using *exp*(y)-0.01. To assess the association between the LC-HRMS and Abraxis ELISA methods, the sample Pearson product-moment correlation coefficient (*r*) was determined using robust Bayesian parameter estimation (Kruschke, 2013).

Five Bayesian analyses of variance (ANOVA) were conducted to determine sources of variability in microcystin concentrations in crocodile tissues. The five models were: a simple intercept model describing the overall median of the 24 sample estimates of microcystin concentration; a group model assessing differences between the good and bad survivorship groups irrespective of tissue type; a tissue type model assessing differences among the four tissue types (liver, egg-shell membrane, unfertilised egg, yolk) irrespective of survivorship group; a main effects (or additive) model assessing both survivorship group and tissue type; an interaction model assessing main effects of survivorship group and tissue type and their respective interactions.

For each Bayesian analysis, the posterior distribution for the parameters of the model was estimated using Markov Chain Monte Carlo (MCMC) methods using R2jags (Su and Yajima, 2015), JAGS (Plummer, 2003), and R (R Core Team, 2015). For each analysis, three chains, 60 000 iterations, a burn in of 1000 iterations per chain, and a thinning rate of 10 were used, to give a total of 17 700 simulations per model from which posterior inference was drawn. Diffuse priors were used in all cases for intercept and slope (*Normal*[$\mu = 0$, $\sigma^2 = 1 \times 10^6$]) and diffuse uniform priors were used for the variance of residual error (Uniform[0, 100]). MCMC chain convergence was assessed using traceplot mixing, autocorrelation plots, and potential scale reduction factors. The 95% highest posterior density intervals (HPDI) were used as credible intervals. The different Bayesian ANOVA models were compared using the deviance information criterion (DIC).

3. Results

3.1 Water samples

When comparing the concentration of microcystins as determined by Abraxis ELISA and LC-HRMS (Table 1) the values were mostly within the same range, although the LC-HRMS analysis only reflects the concentrations of three microcystin variants (MC-LR, MC-RR and MC-YR). Nevertheless, the microcystin concentrations determined with the Abraxis ELISA were consistently higher. The microcystin concentrations determined by LC-HRMS and by the Abraxis ELISA were highly correlated (Fig. 2) with $r = 0.94$ (HPDI = 0.77 to 1.00).

Table 1

Microcystin concentrations (µg L^{-1}) in methanol extracted water samples collected from August 2014 - September 2015 at the commercial crocodile breeding dam as analysed by the Abraxis ELISA and LC-HRMS

Month	Abraxis ELISA[#]	LC-HRMS[†]
August 2014	2.92	0.65
September 2014	4.15	0.59
October 2014	1.56	0.12
November 2014	0.40	< LOD
December 2014	0.24	0.01
January 2015	3.25	2.62
February 2015	7.66	5.08
March 2015	13.92	8.27
April 2015	9.74	4.09

[#] LOD = 0.10 µg L^{-1}

[†]LC-HRMS = only MC-LR, MC-RR. MC-YR; LOD = 0.002 µg L^{-1}

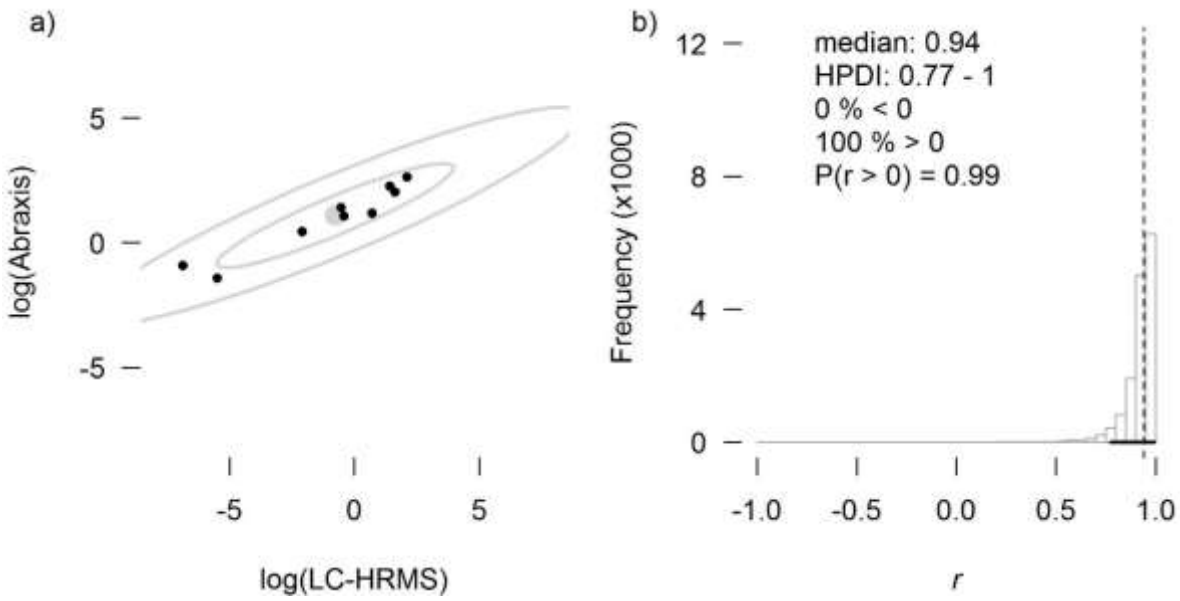

Fig. 2. Correlation of log-transformed microcystin (MC) concentrations measured via methanol extraction between liquid chromatography-high resolution mass spectrometry (LC-HRMS) and the Abraxis ELISA. (a) Model fit of the estimated bivariate t-distribution (ellipses covering 50% and 95% of the distribution) relative to the log-transformed raw data. (b) Bayesian posterior distribution on the sample Pearson product-moment correlation coefficient (*r*). The highest posterior density interval (HPDI) is depicted with the thick horizontal line (b).

3.2 Dead hatchling liver and yolk, egg-shell membranes and unfertilised eggs

Microcystin concentrations (MC-LR, MC-RR, MC-YR) in the crocodile egg and hatchling samples collected from clutches with a good hatching rate ranged between 0 - 1.76 ng g^{-1} where the egg-shell membranes had the highest concentration (Table 2). Microcystin concentrations in samples collected from clutches with a bad hatching rate ranged from 0 – 1.63 ng g^{-1} with the highest concentration detected in the hatchling yolk. However, it should be noted that the percentage recovery rates of spiked samples were low (Table 3).

Table 2

Microcystin (sum of MC-LR, MC-RR and MC-YR) concentrations (ng g^{-1} wet weight [WW]) in pooled crocodile hatchling liver and yolk, crocodile egg-shell membranes and unfertilised crocodile eggs collected during various stages of the hatching period (early, middle, late) for groups of differing survivorship (good, bad). The numbers in brackets represent the number of samples that were pooled.

Sample type	Early		Middle		Late	
	Good	Bad	Good	Bad	Good	Bad
Egg-shell membrane	1.76 (10)	0.53 (10)	0.19 (10)	0.31 (10)	0.73 (10)	0.56 (n=9)
Unfertilised egg	0.17 (4)	0 (5)	0 (5)	0. 04 (5)	0.05 (5)	0.06 (n=5)
Hatchling liver	0.43 (2)	0 (5)	0.55 (4)	1.27 (5)	0 (5)	0 (n=5)
Hatchling yolk	0.17 (2)	1.63 (3)	0.18 (4)	1.48 (5)	0.15 (5)	0 (n=5)

Table 3

Percentage recovery of spiked samples

Sample type	Microcystin LR	Microcystin RR	Microcystin YR
Egg-shell membrane	7.0%	5.8%	4.9%
Unfertilised eggs	16.1%	14.5%	11.3%
Hatchling liver	26.9%	4.4%	15.0%
Hatchling yolk	33.5%	15.2%	15.6%

Of the five Bayesian models used to assess sources of variability in microcystin tissue concentrations, the tissue type model (differences among tissue types irrespective of hatching or survivorship rate) was the best model (Table 4). This model had the lowest (best) deviance information criterion (DIC), which assesses both the fit and the complexity of the models evaluated. However, this model did not perform much better than the null or intercept model, and the DIC provides only a relative assessment among the models evaluated – it does not indicate how well the best model approximates truth.

Table 4

Performance of Bayesian models of variability in microcystin concentrations.

Model	pD[#]	DIC[†]
Tissue type	6.9	100.4
Intercept (null model)	2.4	100.9
Survivorship group	3.8	103.4
Main effects (group and type)	8.7	103.4
Full model with interactions (group*type)	16.0	114.5

[#]pD = Effective number of parameters estimated

[†]DIC = Deviance Information Criterion

The tissue type model suggests that the liver, yolk and unfertilised egg all have similar microcystin concentrations, while the membranes have higher microcystin concentrations (Fig. 3). The microcystin concentration in membranes was 0.47 ng g^{-1} higher than in liver tissue (or a 6.6-fold relative increase). The Bayesian probability of the microcystin concentration in membranes being higher than in liver tissue was 96.5% (a medium to high certainty). The microcystin concentration in membranes was also 0.52 ng g^{-1} higher than in unfertilised eggs (or a 17.8-fold relative increase), and 0.33 ng g^{-1} higher than in fertilised yolks (or a 1.5-fold relative increase).

With increasingly complex models of the variability of microcystin concentrations, the sample sizes within the comparison groups decrease, and hence the variability in the estimates of the effect sizes increases, as do the Bayesian credible intervals around the group medians (Fig. 3).

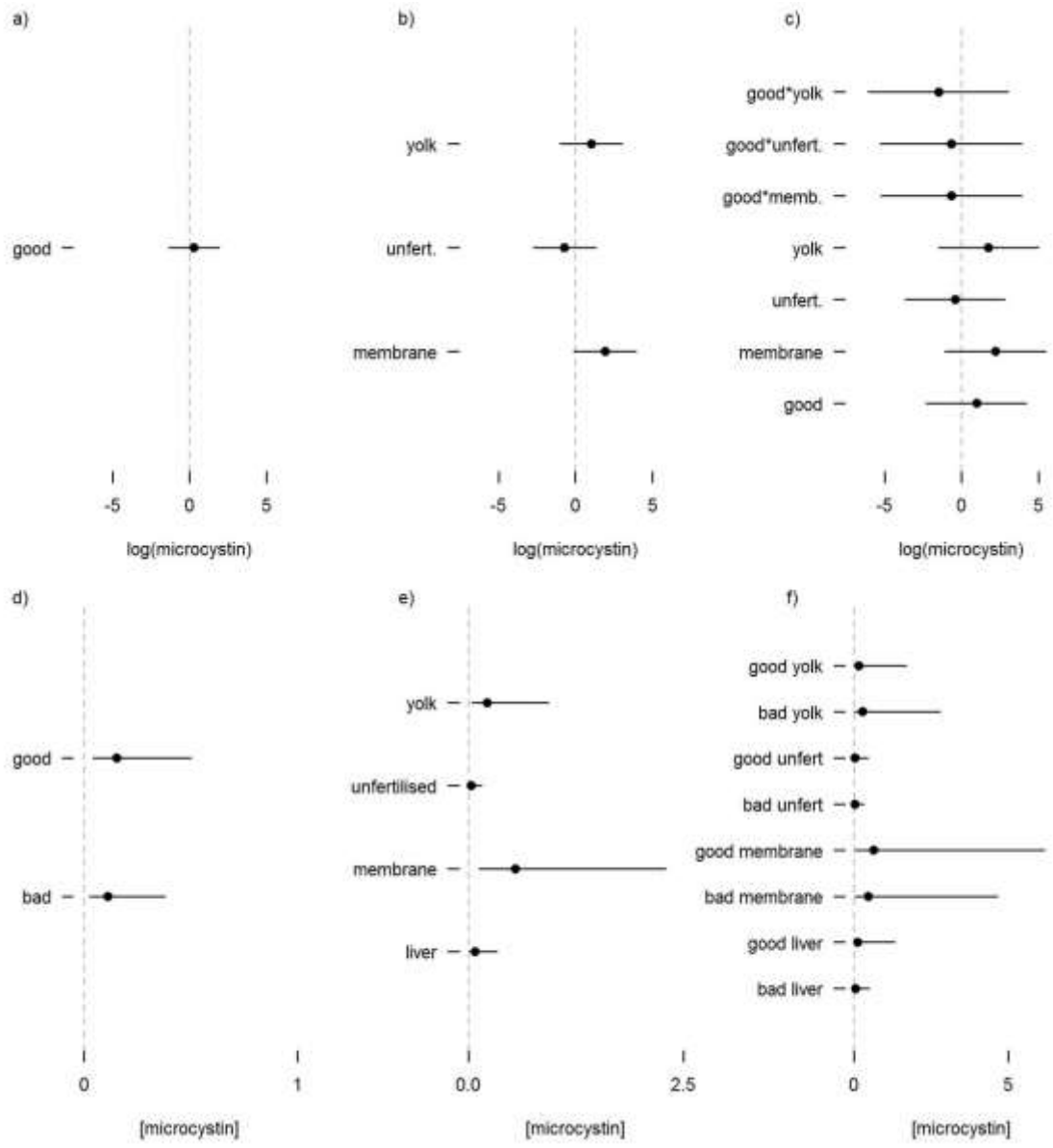

Fig. 3. Factors associated with variability in microcystin concentrations in Nile crocodiles as described by their log-transformed effect sizes (a – c) relative to arbitrarily chosen reference groups, and back-transformed group values (d – f). Effect sizes and group values are expressed as medians and Bayesian 95% credible (highest posterior density) intervals. (a, d) Effect of the good survivorship group relative to the bad surviorship group. (b, e) Effect of different tissue types relative to liver tissue. (c, f) Effect of the interaction between survivorship group and tissue type relative to the bad survivorship group and liver tissue.

4. Discussion

High concentrations of microcystins were recorded in the crocodile farm's breeding dam (Table 1). It appears that the sum of the microcystin (MC-LR, MC-RR, MC-YR) concentration as determined by LC-HRMS analysis is approximately in the same order as the ELISA result. However, the concentrations as determined by ELISA were consistently higher, most probably because it also detects other microcystin variants and even nodularins as well (Fischer et al., 2001). The water level of the crocodile breeding dam is regularly topped-up and replenished with water from the Hartbeespoort Dam. The Hartbeespoort Dam is known notoriously as one of the most eutrophic dams in South Africa, containing various microcystin- synthesising phytoplanktonic species (Ballot et al., 2014). Masango and co-workers (2008) determined microcystin concentrations of 3.7 mg L^{-1} during winter months and 86.1 mg L^{-1} during summer months. Furthermore, water at the crocodile farm's breeding dam may be further nutrient enriched because crocodiles feed in the water, leaving food remnants high in protein content, and crocodiles eliminate body wastes in the water, which will support cyanobacterial growth. Disconcertingly, it should be noted that wild Nile crocodiles are most probably also exposed to microcystins in their habitat (Oberholster et al., 2009; Bengis et al., 2016).

The results presented here confirm that vertical transmission of microcystins from the female crocodile to the eggs does occur. Microcystin variants could be detected in the egg-shell membranes, unfertilised eggs as well as in the liver and yolk of dead hatchlings (Table 2). The data suggest that there were differences in microcystin concentrations among tissue types, with moderate to high certainty.

Chen and co-workers (2009) have reported microcystins in the gonads of another oviparous reptile (a turtle), and in the gonads, egg yolks and egg whites of two egg-laying birds, namely the domestic duck and a heron. This is the first report highlighting microcystin in the eggs of the Nile crocodile. The female crocodiles were exposed to microcystins in the water of the breeding dam during ovulation (August-September) and while the eggs were retained

in the oviduct for about three weeks before nesting (Kofron, 1990). In this study, the yolk of dead crocodile hatchlings contained up to 1.63 ng microcystins (sum of MC-LR, MC-RR and MC-YR) per g wet weight (WW). Relatively high microcystin (MC-LR and MC-YR) concentrations in egg yolk (15 ng g^{-1} dry weight [DW]) of a water bird have also been recorded (Chen et al., 2009).

Several studies have reported toxic effects such as abnormal hatching rates and decrease in survival rates following exposure to microcystins in fish species (Oberemm et al., 1999; Liu et al., 2002; Malbrouck and Kestemont, 2006). A slightly higher microcystin concentration in the egg-shell membranes (0.19 – 1.76 ng g^{-1} WW) relative to the other tissue types (i.e. hatchling liver and yolk and unfertilised eggs) was detected. The egg-shell membrane probably does influence hatching success since it is an outer layer offering protection to the yolk and albumen. If microcystins were able to penetrate through the egg-shell membranes into the yolk it might affect the hatchability of crocodilian eggs.

The tissue concentrations were probably underestimated with the extraction method employed for LC-HRMS as the recovery from spiked samples was very low (Table 3). Ernst et al. (2005) also reported very low recoveries (1 – 3%) from liver samples incubated with 1 µg MC-LR g^{-1}. It is also possible that bound microcystins that may be present in the tissue samples were not extracted (Lance et al., 2010). Even though vertical transmission of microcystins to the Nile crocodile egg occurs its role in the hatching rate of eggs and survivorship of the hatchlings is still uncertain. On the other hand, the low hatching rates and the increased numbers of unfertilised eggs could also potentially be attributed to microcystin-induced infertility in male Nile crocodiles. Daily exposure of male Japanese quails for eight weeks to low concentrations of MC-LR, MC-RR and MC-YR in their feed caused testicular atrophy and sparse spermatozoa (Damkova et al., 2011).

Another interesting observation was the absence of tails in some of the hatchlings (Fig. 4). Although the incidence is not known, developmental abnormalities have been described in fish following exposure to microcystins (Oberemm et al., 1999; Liu et al., 2002; Jacquet et

al., 2004). In an amphibian species, the axolotl (*Ambystoma mexicanum*), 35-day old larvae exposed to microcystins (MC-LR, MC-RR, MC-YR) at 50 µg L^{-1} exhibited less developed forelimbs and some had only three digits (Oberemm et al., 1999) .

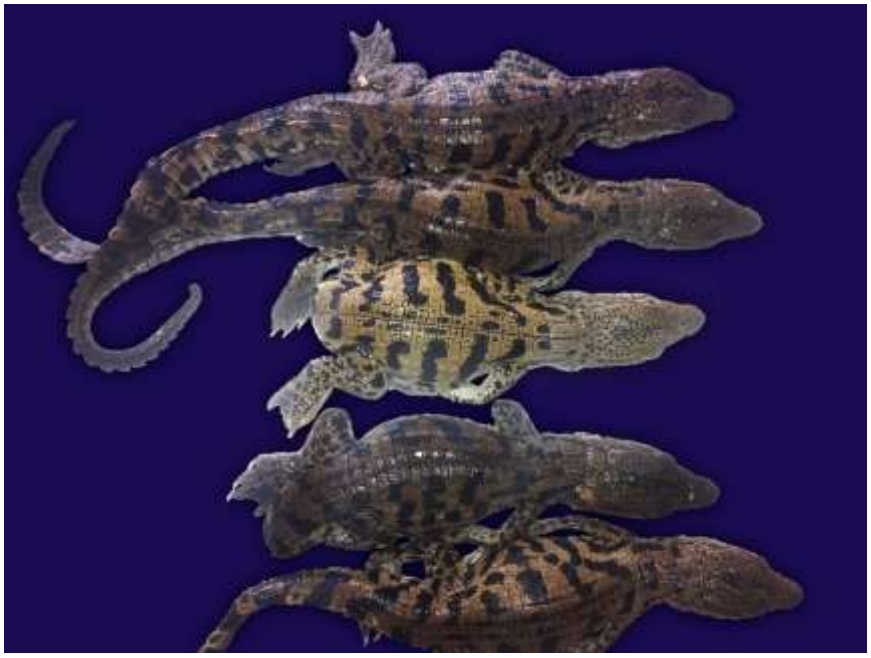

Fig. 4. Dead crocodile hatchlings, two displaying an absence of a tail.

One problem with this study to detect differences in hatching rate and survivorship was the low sample size. Although samples reflect pooled samples from a larger sampling population, the variability within some of the groups remained very high and this combined with the low sample size reduced the available power to detect differences.

Although vertical transmission of microcystins to the Nile crocodile egg occurs, no final conclusion could be drawn as to whether microcystins have a negative effect on the hatchability of crocodile eggs and subsequent survivorship of the hatchlings. There was no difference in microcystin concentrations among good and bad clutches, either across all tissue types or within a specific tissue type. However, the sample sizes in this study were small and hence the power to detect differences in microcystin concentration was low. The information presented here will be used to inform power analyses to guide future studies. To

definitely rule out the role that microcystins play, if any, in the reduced hatching rate of Nile crocodile eggs, a comprehensive study over consecutive breeding seasons at different commercial farms is planned. This is especially important in the light of climate change and global warming contributing to the upsurge in toxic cyanobacterial blooms (Oberholster et al., 2009; Paerl and Huisman, 2009). In addition, the possible adverse consequences of vertical transmission of microcystins to Nile crocodile eggs should be ascertained as free-ranging, wild Nile crocodiles are classified as a protected species of high conservation value in South Africa under the Biodiversity Act, 2004 (NEMBA, 2007) and most probably in other Sub-Saharan African countries as well.

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References

Ashton, P.J., 2010. The demise of the Nile crocodile (*Crocodylus niloticus*) as a keystone species for aquatic ecosystem conservation in South Africa: the case of the Olifants River. Aquat. Conserv. 20, 489-493.

Ballot, A., Sandvik, M., Rundberget, T., Botha, C.J., Miles, C.O., 2014. Diversity of cyanobacteria and cyanotoxins in Hartbeespoort Dam, South Africa. Mar. Freshwater Res. 65, 175-189.

Bengis, R., Govender D., Lane, E., Myburgh, J.G., Oberholster, P.J., Buss, P., Prozesky, L., Keet, D., 2016. Eco-epidemiological and pathological features of wildlife mortality

events related to cyanobacterial biointoxication in the Kruger National Park, South Africa. J. S. Afr. Vet. Assoc. 87, 1-9.

Bruno, M., Melchiorre, S., Messineo, V., Volpi, F., Di Corcia, A., Aragonac, I., Guglielmone, G., Di Paolod, C., Cennid, M., Ferranti, P., Gallo, P., 2009. Microcystin detection in contaminated fish from Italian lakes using ELISA immunoassays and LC-MS/MS analysis. In: Gault, P. M., Marler, H.J. (Eds.), Handbook on Cyanobacteria. Nova Science Publishers, pp. 191-210.

Carmichael, W.W., 1992. Cyanobacteria secondary metabolites – the cyanotoxins. J. Appl. Bacteriol. 72, 445-459.

Chen, J., Zhang, D., Xie, P., Wang, Q., Ma, Z., 2009. Simultaneous determination of microcystin contaminations in various vertebrates (fish, turtle, duck and water bird) from a large eutrophic Chinese lake, Lake Taihu, with toxic *Microcystis* blooms. Sci. Total Environ. 407, 3317-3322.

Combrink, X., Korrûbel, J.L., Kyle, R., Taylor R., Ross P., 2011. Evidence of a declining Nile crocodile (*Crocodylus niloticus*) population at Lake Sibaya, South Africa. S. Afr. J. Wildl. Res. 41, 145-157.

Damkova, V., Paskova, V., Bandouchova, H., Hilscherova, K., Sedlackova, J., Novotny, L., Peckova, L., Vitula, F., Pohanka, M., Pikula, J., 2011. Testicular toxicity of cyanobacterial biomass in Japanese quails. Harmful Algae 10, 612-618.

Ernst, B., Dietz, L., Hoeger, S.J., Dietrich, D.R., 2005. Recovery of MC-LR in fish liver tissue. Environ. Toxicol. 20, 449-458.

Fischer, W.J., Garthwaite, I., Miles, C.O., Ross, K.M., Aggen, J.B., Chamberlin, A.R., Towers, N.A., Dietrich, D.R., 2001. Congener-Independent Immunoassay for Microcystins and Nodularins. Environ. Sci. Technol. 35, 4849-4858.

Flint, N.S., Van der Bank, F.H., Grobler, J.P., 2000. A lack of genetic variation in commercially bred Nile crocodiles (*Crocodylus niloticus*) in the North-West Province of South Africa. Water SA. 26, 105-110.

Jacquet, C., Thermes, V., De Luze, A., Puiseux-Dao, S., Bernard, C., Joly, J., Bourrat, F., Edery, M., 2004. Effects of microcystin-LR on development of medaka fish embryos (*Oryzias latipes*). Toxicon 43, 141-147.

Kofron, C.P., 1990. The reproductive cycle of the Nile crocodile (*Crocodylus niloticus*). J. Zool. 221, 477-488.

Kruschke, J.K., 2013. Bayesian estimation supersedes the *t* test. J. Exp. Psychol. 142, 573-603.

Lance, E., Neffling, M-R., Gérard, C., Meriluoto, J., Bormans, M., 2010. Accumulation of free and covalently bound microcystins in tissues of *Lymnaea stagnalis* (Gastropoda) following toxic cyanobacteria or dissolved microcystin-LR exposure. Environ. Pollut. 158, 674–680.

Lane, E.P., Huchzermeyer, F.W., Govender, D., Bengis, R.G., Buss, P.E., Hofmeyr, M., Myburgh, J.G., Steyl, J.C., Pienaar, D.J., Kotze, A., 2013. Pansteatitis of unknown etiology associated with large-scale Nile crocodile (*Crocodylus niloticus*) mortality in Kruger National Park, South Africa: pathological findings. J. Zoo Wildlife Med. 44, 899-910.

Liu, Y., Song, L., Li, X., Liu, T., 2002. The toxic effects of microcystin-LR on embryo-larval and juvenile development of loach, *Misguruns mizolepis* Gunthe. Toxicon 40, 395-399.

Malbrouck, C., Kesemont, P., 2006. Effects of microcystins on fish. Environ. Toxicol. Chem. 25, 72-86.

Masango, M., Myburgh, J., Botha, C., Labuschagne, L., Naicker, D., 2008. A comparison of *in vivo* and *in vitro* assays to assess the toxicity of algal blooms. Water Res. 42, 3241-3248.

Luxmoore R., 1992. Control of international trade in crocodile products, in: Smith, G.A., Marais, J. (Eds.), Conservation and Utilization of the Nile crocodile in South Africa: Handbook on Crocodile Farming. The Crocodilian Study Group of Southern Africa, pp 1-10

NEMBA - National Environmental Management Biodiversity Act, 2004 (Act 10 of 2004): Publication of Lists of Critically Endangered, Endangered, Vulnerable and Protected Species. Government Gazette, South Africa, 23 February 2007.

Ndlela, L.L., Oberholster, P.J., Van Wyk, J.H., Cheng, P.H., 2016. An overview of cyanobacterial bloom occurrences and research in Africa over the last decade. Harmful Algae 60, 11-26.

Oberemm, A., Becker, J., Codd, G.A., Steinberg, C., 1999. Effects of cyanobacterial toxins and aqueous crude extracts of cyanobacteria on the development of fish and amphibians. Environ. Toxicol. 14, 77-88.

Paerl, H.W., Huisman, J., 2009. Climate change: a catalyst for global expansion of harmful cyanobacterial blooms. Env. Microbiol. Rep. 11, 27–37.

Plummer, M., 2003. Jags: A program for analysis of Bayesian graphical models using Gibbs sampling, in: Proceedings of the 3rd International Workshop on Distributed Statistical Computing. pp 20-22.

R Core Team, 2012. R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna. http://www.R-project.org/ (accessed 3.12.2015)

Reichwaldt, E.S., Ghadouani, A., 2012. Effects of rainfall patterns on toxic cyanobacterial blooms in a changing climate: Between simplistic scenarios and complex dynamics. Water Res. 46, 1372-1393.

Rundberget, T., Gustad, E., Samdal, I.A., Sandvik, M., Miles, C.O., 2009. A convenient and cost-effective method for monitoring marine algal toxins with passive samplers. Toxicon 53, 543-550.

Spoof, L., Catherine, A., 2017. Tables of microcystins and nodularins, in: Meriluoto, J., Spoof, L., Codd, G.A. (Eds.), Handbook of Cyanobacterial Monitoring and Cyanotoxin Analysis. John Wiley and Sons, Chichester, pp. 526-537.

Su, Y.S., Yajima, M., 2015. R2jags: Using R to Run 'JAGS'. http://CRAN.R-project.org/package=R2jags (accessed 3.12.2015)

Trinchet, I., Cadel-Six, S., Djediat, C., Marie, B., Bernard, C., Puiseux-Dao, S., Krys, S. and Edery, M., 2013. Toxicity of harmful cyanobacterial blooms to bream and roach. Toxicon 71, 121-127.

Van As, S., 2016. Crocodile top end stable, more pressure on the middle market. African Leather. 10, 5.

Van Ginkel, C.E., 2003. A national survey of the incidence of cyanobacterial blooms and toxin production in major impoundments. *Internal Report No. N/0000/00/DEQ/0503. Resource Quality Services, Department of Water Affairs and Forestry, Pretoria, South Africa.*