Assessment of selected biochemical parameters and humoral immune response of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*

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Fifteen crocodiles were randomly divided into three groups of five animals. They represented high-infection, medium-infection and low-infection groups of 642 larvae/kg, 414 larvae/kg and 134 larvae/kg bodyweight, respectively. The parameters assessed were blood glucose, creatine phosphokinase (CPK), lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT). The humoral immune response to *Trichinella zimbabwensis* infection was evaluated in all three groups by an indirect ELISA method. The results showed deviations from normal parameters of blood glucose, CPK, LDH, AST and ALT when compared with reported levels in uninfected reptiles. Contrary to studies involving mammals, hypoglycaemia was not observed in the infected groups in this study. Peak values of blood glucose were reached on post-infection (PI) Day 49, Day 42 and Day 35 in the high-infection, medium-infection and low-infection groups, respectively. Peak values of LDH and AST were observed on PI Day 56, Day 49 and Day 42 in the high-infection, medium-infection and low-infection groups, respectively. Peak values of CPK were observed on Day 35 PI in all three groups. Peak ALT values were reached on Day 56 in the high-infection group and on Day 28 PI in both the medium-infection and low-infection groups. No correlations between the biochemical parameters and infection intensity were observed. Peak antibody titres were reached on Day 49 PI in the medium-infection group, and on Day 42 PI in both the high-infection and low-infection groups. Infection intensity could not be correlated with the magnitude of the humoral immune response or time to sero-conversion. Results from this study were in agreement with results reported in mammals infected with other *Trichinella* species and showed that antibody titres could not be detected indefinitely.

Introduction

Apart from their sylvatic and domestic animal hosts, several *Trichinella* species infect humans; the most important species are *Trichinella spiralis* and *Trichinella britovi* (Gottstein *et al*. 2009). Humans become infected through the consumption of raw or undercooked meat from infected animals (Dupouy-Camet 2000; Gottstein *et al*. 2009). The zoonotic importance of *Trichinella* forms the basis for the implementation of measures aimed at the control or eradication of the parasite from the human food chain (Gottstein *et al*. 2009; Mukaratirwa *et al*. 2013). However, despite implementation of control measures, trichinellosis remains a major zoonotic threat in many parts of the world (Mukaratirwa *et al*. 2013; Murrell & Pozio 2011).

Trichinella larvae invade muscle tissue, which causes direct damage to the muscle cell during migration of larvae and indirectly by virtue of the inflammatory response of the host (Bruschi & Chiumiento 2011). This damage also coincides with increased cell membrane permeability and leakage of fluid into the adjacent tissue (Kociecka 2000). This process has been linked to an increase of creatine phosphokinase (CPK), lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT) in the blood (Kociecka 2000).

Serum levels of CPK, LDH and AST are considered to be supplementary in the diagnosis of *Trichinella* infections in humans (Gottstein *et al*. 2009). Jongwutiwes *et al*. (1998) reported elevated levels of CPK, LDH, AST and ALT in human patients infected with *Trichinella pseudospiralis*. Increased serum levels of ALT in pigs infected with *T. spiralis* have also been previously reported (Ribicich *et al*. 2007). However, elevated serum levels of these enzymes are not necessarily indicative of *Trichinella* infection, as there may be other causes (Koudela & Schanzel 1980; Ribicich *et al*. 2007; Srivastava & Chosdol 2007; Tassi *et al*. 1995; Wisniewska 1970). Comparisons between rats and human patients indicated that an increase in enzyme levels is dependent on the individual response from the host rather than being correlated with intensity of infection or clinical severity (Wisniewska 1970).

Normal biochemical values for several crocodile species have been previously reported (Lovely *et al.* 2007; Millan & Janmaat 1997; Padilla *et al.* 2011; Stacy & Whitaker 2000) but to the authors' knowledge, no studies have been conducted to investigate the effect of *Trichinella* infection on the biochemical parameters of Nile crocodiles.

Trichinella infection has also been reported to influence blood glucose levels, and hypoglycaemia has been reported in humans (Busila *et al.* 1968), mice (Nishina & Suzuki 2002; Wu *et al.* 2009) and dogs (Reina *et al.* 1989) infected with *Trichinella* species. The decrease in blood glucose has been attributed to the depletion of blood glucose by the parasite larvae (Wu *et al.* 2009). In a study involving mice infected with *T. spiralis*, hypoglycaemia was observed at 10 days post infection (PI) (Nishina & Suzuki 2002).

The cuticle of nematodes contains immune-specific antigens targeted by the host immune system (Phillip *et al.* 1981). Larvae and adults of the genus *Trichinella* are antigenically heterogeneous (Fabre *et al.* 2009). Enzyme linked immunosorbent assay (ELISA) is a commonly used method to measure the humoral immune response of the host to the antigen. The method relies on the metabolic excretory/secretory antigens (ESA) comprising of related glycoproteins that are released by the larvae (Gottstein *et al.* 2009). An important carbohydrate epitope, tyvelose, which is responsible for the induction of the humoral immune response, is situated on the TSL-1 antigen contained within stichocyte cells of the cuticle; a synthetic variant of this carbohydrate is used in the ELISA (Gottstein *et al.* 2009). Although highly specific, the use of synthetic tyvelose antigen in the ELISA is less sensitive than ESA (Gottstein *et al.* 2009).

Information is scant about the antibody response of the host against muscle stages of the parasite, but a mixed isotype response of Immunoglobulin G (IgG) 1, IgG2 and IgE has been reported in chronic infections, with IgG1 being the most dominant (Fabre *et al.* 2009). The use of serological tests as diagnostic tools in animal trichinellosis has previously been evaluated. Enzyme immunoassay tests (Gamble *et al.* 1996; Soule *et al.* 1989) and indirect immunofluorescence assays (Reina *et al.* 1996; Soule *et al.* 1989) have been conducted in goats and horses. Immunoassays were reported to be useful in horses but the study animals were all euthanased at 12 weeks post infection (Gamble *et al.* 1996), thus the persistence of antibodies beyond this time frame was never investigated. Similar results were also obtained in a study involving goats (Reina *et al.* 1996). In a study by Pozio *et al.* (2002), the immune response of horses against *T. spiralis* and *Trichinella murrelli* was evaluated for four-to-five months following infection using both ELISA and Western Blot techniques. In most cases, specific IgG was detectable only between three to seven weeks PI and maximum persistence of antibodies was recorded at 33 weeks PI in one horse infected with 20 000 *T. murrelli* larvae. Western Blot analysis and ELISA were also developed for pigs and, although relatively high specificity and sensitivity of the tests were reported (Nöckler *et al.*

2009), samples were not collected over extended periods to evaluate the persistence of antibodies. The aforementioned studies showed the practical application of these techniques to be limited. Specific antibodies against *Trichinella* do not persist indefinitely and can only be detected for limited periods following infection. Since *Trichinella zimbabwensis* is a non-encapsulated species, it is expected that the host immune response is stronger and more persistent due to the direct contact between the parasite larvae and host tissue (Huchzermeyer, F.W., pers. comm., 2008). An experimental study to determine the feasibility of the use of ELISA for the detection of *T. zimbabwensis* infection in crocodiles indicated that antibody titres decreased and eventually disappeared altogether (Ludovisi *et al.* 2013).

The objective of this study was to evaluate the effect of *T. zimbabwensis* infection intensity on the levels of blood glucose, AST, ALT, CPK and LDH and on the humoral immune response of experimentally infected crocodiles.

Materials and methods
Source of study animals

Fifteen 7-year-old Nile crocodiles (13 males and 2 females) within the size range of 1.35 m – 1.8 m in length were used in the present study. The crocodiles represented a group of animals not intended for commercial purposes and sourced from a farmed population with no history of *T. zimbabwensis* infection prior to commencement of the study. The animals were captured on the Wilderness Roads farm in Low's Creek, Mpumalanga Province, South Africa. They were immobilised with 0.4 mL Gallamine triethiodide (40 mg/mL) (Kyron, Johannesburg, South Africa), which was injected intramuscularly on the lateral aspect of the tail base of each animal before being transported to the experimental housing.

Pre-trial data collected from the animals included weight, sex and length of the animals. Clipping the horizontal scutes following a specific pattern on the tail identified each animal. For the high-infection group, both the left and right scutes were clipped in sequence according to the number assigned to the animal. For the low-infection and medium-infection groups, only the scutes on the left or right were clipped, respectively. The experiment was carried out from January 2012 to March 2012, when climatic conditions ensured good feeding and optimal physical condition of the animals.

Animal husbandry and feeding

Animal husbandry and feeding practices for the study animals were followed, as described by the South African National Standard for crocodiles in captivity SANS 631:2009 (SABS Standards Division 2009).

The study animals were housed on a smallholding belonging to the Mpumalanga Tourism and Parks Agency (MTPA) on

the outskirts of Nelspruit, Mpumalanga province, South Africa. A fenced enclosure (10 m x 5 m) was constructed using 65 mm diamond mesh fencing, with a single-access gate. The enclosure allowed for a temperature gradient in the water to provide optimal thermoregulation (La Grange & Mukaratirwa 2014). During capture and sampling the pond was drained weekly and replenished with fresh water. Animals were fed with coarsely minced commercially bred chicken carcasses enriched with vitamin and mineral components (Feedmix, Johannesburg, South Africa) that were supplemented as 2.50 kg and 1.25 kg, respectively, in 1000 kg of wet ration.

The study animals were fed approximately 10 kg of food two to three times a week; no food was offered on days immediately prior to testing.

Source and preparation of infective material

As part of a previous study, *T. zimbabwensis*-infected meat was sourced from a crocodile experimentally infected with *T. zimbabwensis* (Ludovisi *et al.* 2013). Muscle tissue was collected from various sites, minced and thoroughly mixed using a ladle. One hundred grams of the homogenised sample was subjected to artificial digestion according to the method of Nöckler and Kapel (2007) and infection level was determined to be 30 larvae per gram (LPG) of the homogenised sample. Infective material for each individual animal was calculated and separately packaged and refrigerated at 4 °C until the day of infection.

Infection of study animals

The animals were manually restrained and the infective dose was administered through a stomach tube. The high-infection group of crocodiles were infected with an average of 642 larvae/kg of bodyweight, the medium-infection group with an average of 414 larvae/kg of bodyweight and the low-infection group with an average of 134 larvae/kg of bodyweight. The animals were continuously monitored for at least 30 min following infection for side effects or regurgitation of infective material.

Collection of blood and sera

Blood was collected from each of the animals weekly from the date of infection (Day 0) until eight- weeks post infection (Day 56) to test for blood glucose and levels of CPK, LDH, ALT and AST. A 10 mL syringe and a 21-gauge needle were used to collect blood from the supra-vertebral sinus; approximately 8 mL blood was collected weekly from each animal. In order to separate the serum, blood was allowed to clot and the sample was centrifuged at 10 000 rpm for 15 min.

Preservation of samples

For the detection of antibodies, 2 mL serum from each animal was transferred to sterile cryotubes with screw caps and preserved in 0.01% merthiolate solution. The preserved samples were stored at 4 °C until completion of the trial. The remaining sera were frozen at -18 °C and used for the enzyme assays.

Testing of samples

Blood glucose was tested immediately after collection using an Accu-Chek® Active (Roche Diagnostics, Indianapolis, USA) glucometer to minimise the impact of stress (Smith & Marais 2004).

Sera preserved in 0.01% merthiolate were referred to the International *Trichinella* Reference Centre in Rome, Italy, for testing according to procedure based on Ludovisi *et al.* (2013).

Sera frozen at -18 °C were submitted to the biomedical research laboratory at the University of KwaZulu-Natal, Westville campus, Durban, South Africa, for testing. The samples were tested for LDH, ALT, AST and CPK enzyme levels. An automated chemistry analyser, Labmax Plenno (Lagoa Santa, Costa Brava, Brazil) was used for the analyses in accordance with manufacturer's recommendations.

Data analysis

Every week, mean blood glucose and serum concentrations of CPK, LDH, ALT and AST were determined for each group of animals over the trial period. Mean serum concentrations of biochemical parameters on Day 0 of the trial were compared, where possible, with normal reference ranges reported for Nile crocodiles (Botha 2010; Huchzermeyer 2003; Lovely *et al.* 2007; Padilla *et al.* 2011). Alternatively, where normal reference ranges for Nile crocodiles could not be found, comparisons were made with reference ranges reported for other crocodilian species (Diethelm & Stein 2006; Millan & Janmaat 1997; Padilla *et al.* 2011) (Table 1). Values from Day 7 PI were expressed as a percentage of the normal values recorded on Day 0 and calculated as follows:

$$Pv = (Iv/D0)*100 \qquad \text{[Eqn 1]}$$

Where Pv is the new percentage value, Iv is the initial value on the specific day and D0 represents the value on Day 0. Data were log transformed ($\log_{10}[x+1]$) and analysis of variance (IBM SPSS Statistics 19) was used to compare results from the different groups. Non-parametric, bivariate correlation (Spearman's rho) analyses (IBM SPSS Statistics 19) were conducted in order to determine relationships between biochemical parameters and infection dose.

Results
Blood glucose

The mean concentration of blood glucose for the three experimental groups is shown in Table 2. In the high-infection and medium-infection groups, initial peaks in blood glucose values were observed on Day 14 PI and Day 7 PI, respectively. This was followed by a slight decrease the following week. Analysis of variance revealed significant differences in blood glucose concentrations on Day 28 PI between the high-infection and low-infection groups and between the medium-infection and low-infection groups

TABLE 1: Biochemical ranges of *Crocodylus niloticus* and reference ranges reported in other crocodilian species.

Species	Glucose	Alanine transaminase (ALT)	Aspartate transaminase (AST)	Creatine phosphokinase (CPK)	Lactate dehydrogenase (LDH)	Reference
Crocodylus niloticus	3.80 mmol/L – 7.50 mmol/L	8.00–59.00	16.0–49.0	61.4–1893.5	638.0–3173.0	Current study
	81.60 mg/dL	-	-	-	-	Padilla *et al*. (2011); Huchzermeyer 2003
	3.30 mmol/L – 4.80 mmol/L	15.00–69.00	14.0–211.0	-	-	Lovely *et al*. (2007)
	3.87 mmol/L – 5.68 mmol/L	13.00–30.00	24.0–47.0	-	-	Botha (2010)
Alligator mississippiensis	92.00 mg/dL	46.05[a,b]	223.5[b]	-	-	Padilla *et al*. (2011); Huchzermeyer (2003)
	0.00 mg/dL – 198.00 mg/dL	0.00–154.00	0.0–700.0	0.0–8620.0	0.0–2000.0	Diethelm and Stein (2005)
	74.00 mg/dL	-	-	-	-	Stacey and Whittaker (2000)
Paleosuchus palpebrosus	29.00 mg/dL – 187.00 mg/dL	24.00–93.00	42.0–221.0	37.0–9890.0	80.0–6615.0	Diethelm and Stein (2005)
Crocodylus moreletti	64.25 mg/dL – 74.85 mg/dL	16.68–23.72	-	-	-	Padilla *et al*. (2011)
	52.95 mg/dL	-	-	-	-	Huchzermeyer (2003)
Crocodylus porosus	4.50 mmol/dL – 12.10 mmol/dL[c,d,e]	11.00–51.00[c,d]	23.0–157.0[c]	-	-	Millan and Janmaat (1997); Padilla *et al*. (2011)
	4.80 mmol/L – 11.99 mmol/L	12.00–50.00	23.0–148.0	-	-	Richardson *et al*. (2002)
	81.00 mg/dL – 218.00 mg/dL	11.00–51.00	23.0–157.0	-	-	Stacey and Whittaker (2000)
Caiman latirostris	81.82 mg/dL	-	-	-	-	Huchzermeyer (2003)
Crocodylus palustris	55.00 mg/dL – 110.00 mg/dL	-	-	-	-	Huchzermeyer (2003)
	48.00 mg/dL – 110.00 mg/dL	28.00–97.00	23.0–70.0	-	-	Stacey and Whittaker (2000)
Tomistoma schlegelii	75.30 mg/dL	-	-	-	426.2	Huchzermeyer (2003)
Crocodylus mindorensis	60.00 mg/dL – 168.00 mg/dL	-	-	-	-	Stacey and Whittaker (2000)
Crocodylus acutus	101.00 mg/dL	-	-	-	-	Stacey and Whittaker (2000)

[a], Padilla *et al*. (2011); [b], Huchzermeyer (2003); [c], Millan and Janmaat (1997); [d], Padilla *et al*. (2011); [e], Huchzermeyer (2003).
Note: Please see the full reference list of the article, La Grange, L.J. & Mukaratirwa, S., 2014, 'Assessment of selected biochemical parameters and humoral immune response of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*', *Journal of the South African Veterinary Association* 85(1), Art. #1085, 10 pages. http://dx.doi.org/10.4102/jsava.v85i1.1085

TABLE 2: Mean blood glucose levels (mmol/L) in Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*.

Infected groups	Blood glucose levels	Days post infection								
		Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
High infection† (643 larvae/kg bodyweight)	Mean ± SE	4.62 ± 0.33	6.46 ± 0.98	7.24 ± 1.17	4.98 ± 0.37	5.30 ± 0.66	7.54 ± 1.85	8.32 ± 1.09	8.40 ± 1.00	6.76 ± 0.75
	Range	3.80–5.50	3.70–9.70	5.20–11.30	4.20–6.30	3.40–7.30	4.70–14.70	6.80–12.60	4.90–11.10	5.40–10.3
Medium infection† (414 larvae/kg bodyweight)	Mean ± SE	5.32 ± 0.38	7.48 ± 0.67	5.86 ± 0.53	6.28 ± 0.34	5.24 ± 0.49	7.44 ± 0.27	8.98 ± 0.44	6.70 ± 0.31	8.72 ± 0.76
	Range	4.70–6.80	5.50–9.40	4.60–7.80	5.40–7.40	3.80–6.80	6.90–8.40	8.00–10.30	5.50–7.30	6.20–10.4
Low infection† (134 larvae/kg bodyweight)	Mean ± SE	6.06 ± 0.66	5.68 ± 0.57	5.88 ± 0.31	6.42 ± 0.70	8.70 ± 0.30	9.80 ± 1.18	7.34 ± 0.46	9.70 ± 0.99	6.66 ± 0.46
	Range	4.60–7.50	4.40–7.50	4.80–6.70	5.30–9.10	7.80–9.30	5.20–11.70	6.20–8.40	6.90–12.70	5.50–8.20

†, $n = 5$.

TABLE 3: Mean alanine transaminase levels (IU/L) of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*.

Infected groups	Alanine transaminase levels	Days post infection								
		Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
High infection† (642 larvae/kg bodyweight)	Mean ± SE	11.67 ± 2.33	21.00 ± 3.24	21.80 ± 7.11	28.40 ± 5.52	36.20 ± 4.12	36.20 ± 5.15	30.40 ± 11.12	38.50 ± 14.05	64.60 ± 32.30
	Range	8.00–16.00	14.00–32.00	5.20–11.30	4.20–6.30	3.40–7.30	4.70–14.70	6.80–12.60	4.90–11.1	5.40–10.30
Medium infection† (414 larvae/kg bodyweight)	Mean ± SE	35.50 ± 1.85	38.25 ± 4.85	40.80 ± 4.60	42.40 ± 4.80	47.75 ± 6.13	38.40 ± 4.25	47.00 ± 4.09	40.60 ± 3.43	43.20 ± 5.28
	Range	32.00–40.00	25.00–46.00	25.00–50.0	28.00–54.0	39.00–65.0	27.00–51.00	36.00–61.00	28.00–48.0	25.00–58.00
Low infection† (134 larvae/kg bodyweight)	Mean ± SE	33.40 ± 6.82	37.40 ± 7.92	37.60 ± 4.79	44.20 ± 7.15	47.20 ± 4.42	43.60 ± 7.65	44.20 ± 6.02	46.80 ± 10.33	38.80 ± 8.75
	Range	23.00–59.00	28.00–69.00	22.00–48.00	23.00–68.00	32.00–57.00	14.00–57.00	25.00–55.00	8.00–69.0	12.00–59.00

†, $n = 5$.

($p < 0.05$). No correlation was observed between blood glucose and infection dose.

Alanine transaminase

Mean values of ALT for the three experimental groups are shown in Table 3. The highest increase of ALT was observed in the high-infection group on Day 49 PI and Day 56 PI, and it was 42% higher than the initial concentrations on Day 0. Increases in ALT in the medium-infection and low-infection groups were less pronounced and reached peak values of 8% and 11% higher than Day 0 at Day 28 PI. Analysis of variance showed no significant difference ($p > 0.05$) in the increases in ALT concentrations among the groups. However, mean ALT concentrations

were correlated with mean blood glucose concentrations in the high infection group ($R^2 = 0.9$, $p < 0.05$).

Aspartate transaminase

Mean values of AST for all three experimental groups are shown in Table 4. Maximum values were reached on Day 56 PI in the high-infection group. In the medium-infection group, AST values peaked on Day 49 PI and maximum values of AST were reached in the low-infection group on Day 42 PI. On Day 42 PI, the percentage increase of AST in the low-infection group was significantly higher than that observed in the medium-infection group ($p < 0.05$). Analysis of variance showed mean AST values in the low-infection group to be significantly higher on Day 14 PI and Day 42 PI ($p < 0.05$) compared with the high-infection group.

Creatine phosphokinase

The mean values of CPK for all three experimental groups are shown in Table 5. The low-infection group showed significantly lower values of CPK from Day 0 compared with the high-infection and medium-infection groups. Analysis of variance showed significant differences in changes of mean CPK values between the high-infection and low-infection groups from Day 0 until Day 14 of the trial. The percentage increases in CPK values in the low-infection group was significantly higher ($p < 0.05$). Mean CPK values

were significantly increased in the low-infection group compared with the medium-infection group on Day 7 PI, Day 42 PI, and Day 56 PI ($p < 0.05$). Similarly, CPK in the low-infection group increased to significantly higher levels than those recorded for the high-infection group on Day 42 PI and Day 56 PI ($p < 0.05$). The mean increase in CPK value was also significantly higher in the medium-infection group compared with the high-infection and low-infection groups on Day 28 PI ($p < 0.05$). Mean CPK values were significantly correlated with both initial infection dose and mean blood glucose ($R^2 = 0.9$, $p < 0.05$) in the high-infection group, but a significantly negative correlation was observed with overall infection dose in this group ($R^2 = -0.9$, $p < 0.05$). Mean CPK was negatively correlated with mean blood glucose in the medium-infection group ($R^2 = -0.9$, $p < 0.05$). No significant correlation could be established between infection dose and peak CPK values.

Lactate dehydrogenase

The values of LDH for all three experimental groups are shown in Table 6. No correlation could be established between peak LDH values and infection dose. Mean increase in LDH was significantly higher in the low-infection group on Day 28 PI and Day 35 PI compared with the medium-infection group ($p < 0.05$). Mean increases in LDH values were significantly higher in the low-infection group compared to the high-infection group on Day 35 PI

TABLE 4: Mean aspartate transaminase levels (IU/L) of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*.

Infected groups	Alanine transaminase levels	Days post infection								
		Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
High infection† (642 larvae/kg bodyweight)	Mean ± SE	21.75 ± 4.13	18.80 ± 1.28	20.00 ± 0.82	19.00 ± 2.53	20.80 ± 1.11	26.80 ± 5.44	18.75 ± 5.76	21.00 ± 1.92	53.20 ± 3.50
	Range	16.00–34.00	15.00–22.00	18.00–22.00	14.00–26.00	17.00–24.00	9.00–42.00	6.00–32.00	14.00–25.00	44.00–65.00
Medium infection† (414 larvae/kg bodyweight)	Mean ± SE	38.50 ± 4.70	29.75 ± 7.55	25.00 ± 2.59	26.40 ± 1.03	29.50 ± 3.86	28.20 ± 4.04	28.20 ± 2.94	74.00 ± 16.71	31.40 ± 2.58
	Range	27.00–48.00	15.00–48.00	19.00–33.00	24.00–30.00	21.00–37.00	18.00–37.00	19.00–34.00	20.00–120.00	26.00–39.00
Low infection† (134 larvae/kg bodyweight)	Mean ± SE	34.80 ± 4.07	31.40 ± 4.88	35.80 ± 3.34	30.60 ± 3.49	34.80 ± 3.87	39.40 ± 4.83	57.80 ± 8.09	33.60 ± 7.59	24.20 ± 4.09
	Range	26.00–49.00	13.00–41.00	28.00–47.00	23.00–68.00	22.00–40.00	27.00–55.00	38.00–80.00	6.00–52.00	12.00–33.00

†, $n = 5$.

TABLE 5: Mean creatine phosphokinase levels (IU/L) of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*.

Infected groups	Creatine phosphokinase levels	Days post infection								
		Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
High infection† (642 larvae/kg bodyweight)	Mean ± SE	698 ± 145	770 ± 51	872 ± 268	599 ± 185	1507 ± 770	3343 ± 1205	2252 ± 522	1547 ± 874	1884 ± 673
	Range	405–1099	577–854	454–1898	115–1084	269–4534	695–5064	1256–3652	414–5026	524–4410
Medium infection† (414 larvae/kg bodyweight)	Mean ± SE	1209 ± 230	1042 ± 129	802 ± 115	896 ± 201	802 ± 75	3377 ± 1029	1843 ± 539	1681 ± 701	998 ± 187
	Range	448–1294	691–1384	541–1225	391–1449	592–1001	1701–6941	633–3684	690–4448	567–1684
Low infection† (134 larvae/kg bodyweight)	Mean ± SE	93 ± 12	136 ± 41	148 ± 37	162 ± 34	187 ± 29	722 ± 128	297 ± 57	112 ± 20	63 ± 8
	Range	61–123	54–254	75–275	86–271	148–302	266–1016	194–495	55–166	41–86

†, $n = 5$.

TABLE 6: Mean lactate dehydrogenase levels (IU/L) of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*.

Infection	Lactate dehydrogenase levels	Days post infection								
		Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
High infection† (642 larvae/kg bodyweight)	Mean ± SE	827 ± 137	876 ± 184	1256 ± 271	764 ± 92	841 ± 55	1637 ± 318	1521 ± 367	1960 ± 375	4281 ± 240
	Range	563–1348	288–1393	641–2180	455–991	752–1049	1181–2901	991–2959	1045–3021	3811–5194
Medium infection† (414 larvae/kg bodyweight)	Mean ± SE	1140 ± 320	1742 ± 321	1101 ± 157	1246 ± 324	2642 ± 704	1849 ± 392	2532 ± 469	5743 ± 1466	1395 ± 290
	Range	724–2413	1011–2855	489–1360	409–2357	1033–4940	1156–3216	1223–3674	1004–9726	868–2527
Low infection† (134 larvae/kg bodyweight)	Mean ± SE	1592 ± 476	905 ± 158	944 ± 86	2179 ± 481	1729 ± 377	2416 ± 533	4853 ± 750	1532 ± 299	533 ± 191
	Range	638–3173	344–1243	760–1179	1069–3737	915–2726	544–3793	3335–7403	704–2313	275–765

†, $n = 5$.

TABLE 7: Mean change* in anti-Trichinella IgG in sera of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*.

Infected Groups	Immunoglobulin G	Days post infection								
		Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
High infection† (642 larvae/kg bodyweight)	Mean ± SE	0.79 ± 0.46	0.56 ± 0.06	0.67 ± 0.06	0.52 ± 0.04	1.68‡ ± 0.34	1.14 ± 0.40	1.97‡ ± 0.44	0.90 ± 0.21	1.95‡ ± 0.40
	Range	0.67–0.93	0.37–0.69	0.45–0.78	0.52–0.65	0.84–2.68	0.50–2.62	0.86–3.34	0.61–1.73	1.11–3.25
Medium infection† (414 larvae/kg bodyweight)	Mean ± SE	0.91 ± 0.18	0.90 ± 0.09	0.87 ± 0.08	1.42‡ ± 0.19	1.68‡ ± 0.28	1.40‡ ± 0.28	1.21 ± 0.20	2.37‡ ± 0.46	1.87‡ ± 0.36
	Range	0.20–1.13	0.60–1.15	0.57–0.98	0.94–2.05	1.08–3.07	0.57–2.27	0.78–1.98	0.82–3.15	0.72–2.95
Low infection† (134 larvae/kg bodyweight)	Mean ± SE	0.89 ± 0.04	0.94 ± 0.11	0.92 ± 0.15	0.81 ± 0.11	1.62‡ ± 0.38	2.29‡ ± 0.47	2.64‡ ± 0.30	1.41‡ ± 0.50	1.59‡ ± 0.22
	Range	0.73–0.97	0.54–1.22	0.63–1.37	0.53–1.14	0.96–2.94	0.94–3.35	1.60–3.47	0.67–3.32	1.22–2.41

*, Values displayed represent optical density values at 450 nm.
†, $n = 5$; ‡, Values > 1.4 are considered positive.

($p < 0.05$). Mean LDH concentrations in the low-infection group reached significantly higher values on Day 21 PI and Day 42 PI compared with the high-infection group ($p < 0.05$). Mean increase in values of LDH was significantly higher in the high-infection group on Day 56 PI compared with the medium-infection and low-infection groups ($p < 0.05$). LDH values were also significantly higher in the medium-infection group compared with the high-infection group on Day 28 PI and Day 49 PI ($p < 0.05$). Mean LDH values were also significantly higher in the medium-infection group on Day 56 PI compared with the low-infection group ($p < 0.05$). Mean LDH values were negatively correlated with blood glucose in the medium-infection group ($R^2 = -0.9$, $p < 0.05$). A significant correlation was also observed between mean LDH concentrations and infection dose in the low-infection group ($R^2 = 0.9$, $p < 0.05$).

Indirect enzyme immnunosorbent assay

Sero-conversion was observed in all of the experimental animals. Analysis of variance showed no significant differences in the titre levels between the three groups, despite the fact that mean optical density (OD) values in the high-infection group were lower compared with the medium-infection group. Mean OD values were also lower in the medium-infection group compared with the low-infection group. No significant correlation between infection dose and magnitude of the humoral immune response could be established. Peak antibody titres were reached at Day 42 PI in both the high-infection and low-infection groups, whereas the medium-infection group reached its highest value on Day 49 PI. Analysis of variance, however, showed significant differences between the groups on Day 7 PI, Day 21 PI and Day 42 PI. On Day 7, OD values were significantly lower in the high-infection group ($p < 0.05$) compared with medium-infection and low-infection groups. On Day 21, OD values in both the high-infection and low-infection groups were significantly lower than in the medium-infection group ($p < 0.05$). On Day 42, OD values in the low-infection group were significantly higher compared with the high-infection group. However, no significant correlation could be established between infection dose and time to sero-conversion.

Ethical consideration

Ethical approval for the study was obtained through the Animal Ethics Research Committee of the University of KwaZulu-Natal (reference number 035/12/Animal). Where applicable, ARRIVE guidelines for reporting *in vivo* animal experiments (Kilkenny *et al*. 2010) have been adhered to.

Discussion

Mean blood glucose ranges of the experimental crocodiles on Day 0 compared favourably with normal reference ranges reported for Nile crocodiles (Botha 2010; Huchzermeyer 2003; Lovely *et al*. 2007; Padilla *et al*. 2011). Only four animals in this study showed blood glucose values higher than those reported by previous authors, but they still fell within the ranges reported for estuarine crocodiles *Crocodylus porosus* (Millan & Janmaat 1997; Padilla *et al*. 2011).

Wu *et al*. (2009) reported an initial drop in blood glucose levels between Day 8 PI and Day 28 PI in mice experimentally infected with *T. pseudospiralis* and *T. spiralis*. Interestingly, the results reported by Wu *et al*. (2009) showed that the lowest levels of blood glucose in mice were reached at Day 13 PI and Day 18 PI for *T. spiralis* and *T. pseudospiralis*, respectively; *T. pseudospiralis*-infected mice took longer to return to normal. Similarly, mice infected with *T. spiralis* in other studies showed hypoglycaemia at Day 10 PI (Nishina & Suzuki 2002; Nishina *et al*. 2004). The initial decrease in blood glucose observed on Day 7 PI in the low-infection group in the present study is consistent with results obtained from mice experimentally infected with *T. spiralis* (Nishina & Suzuki 2002). In contrast, blood glucose levels increased between Day 0 PI and Day 7 PI in the high-infection group and between Day 0 PI and Day 14 PI in the medium-infection group in the present study. This suggests that the level or intensity of infection has an effect on blood glucose levels. Modulation of the insulin pathway by the parasite is considered to cause hypoglycaemia, and blood glucose levels correspond to larval growth (Wu *et al*. 2009). Wu *et al*. (2009) additionally reported that minimum blood glucose levels in mice experimentally infected with *T. spiralis* and *T. pseudospiralis* were reached on Day 13 PI and Day 18 PI, respectively; in the case of the latter, recovery time from hypoglycaemia was longer. These differences suggest that parasite-specific mechanisms are responsible. However, the initial rise in blood glucose levels observed in the high-infection and medium-infection groups in this study cannot be explained. In the study by Wu *et al*. (2009), blood glucose levels of the infected mice steadily increased following their initial drop and reached almost normal levels by Day 48 PI; they rarely reached levels above normal. In this

study, however, blood glucose levels were maintained above the initial levels recorded on Day 0 and never decreased to normal levels during the experimental period. This phenomenon cannot be explained, but it could be speculated that crocodiles possess physiological adaptations that allow them to up-regulate blood glucose concentrations in accordance with the existing demand.

Dolphins have been reported to switch between a normal and hyperglycaemic state when presented with daily feeding and fasting routines (Venn-Watson *et al*. 2011). This adaptation is necessary to enable these animals to maintain high blood glucose levels, as demanded by their physiology (Venn-Watson *et al*. 2011). The elevated blood glucose levels observed in this study may also have been influenced by stress associated with capture and restraint (Smith & Marais 2004), but the observed variation in responses between the experimental groups suggests that the effects of infection superseded those caused by other stressors. However, the increase in blood glucose levels observed in the medium-infection and high-infection groups between Day 28 PI and Day 42 PI, and Day 21 PI and Day 35 PI in the low-infection group, may be due to the arrival and infiltration of new-born larvae in the muscle tissue. In this study, minimum and maximum blood glucose levels were not reached at the same time in the three groups, but were marked by a one-week delay between each group; the low-infection group reached the high levels first. Studies with mice and reptiles infected with *T. pseudospiralis* and *T. zimbabwensis*, respectively, showed larvae of these two species to be larger in poikilotherms than in mammalian hosts (Pozio *et al*. 2004). This suggests that the metabolic rate of the host may influence larval growth (Pozio *et al*. 2004).

If the growth rate and, subsequently, the metabolic rate of *T. zimbabwensis* can be altered as a result of variation in host metabolism, similar alterations may be caused in accordance with the availability of nutrients, which may explain the delays observed between the experimental groups of this study. Another hypothesis could be that this strategy is employed by the parasite to prevent the sudden release of large numbers of larvae into the host circulation. Large larval burdens in the blood circulation could potentially restrict blood flow in vital organs or produce acute anaphylaxis causing host fatality, which ultimately is detrimental to survival of the parasite.

Mean ALT of the experimental crocodiles compared favourably on Day 0, with normal reference ranges reported for Nile crocodiles (Botha 2010; Lovely *et al*. 2007). Only one animal in this study displayed ALT concentrations lower than those reported by previous authors, but still fell within the range reported for American alligators (*Alligator mississippiensis*) (Diethelm & Stein 2006). Increased levels of ALT are normally more indicative of liver disease and, according to Srivastava and Chosdol (2007), increase in the levels of this enzyme is rarely noted in other cases.

Peak values of ALT were not reached simultaneously in the three groups. Increases in ALT concentrations were less pronounced than those of CPK and LDH, suggesting that ALT is less sensitive to the effects of the parasite on host tissue and that significant increases in this enzyme may only occur in cases of high infection intensity that result in severe tissue damage. This supports the specific nature of this enzyme as an indicator of liver disease rather than muscle damage. Assessment of the individual crocodile results additionally showed high variability within the three groups; this further suggests that the effect of individual host physiology on ALT concentrations supersedes the effect of parasite invasion, especially in lower level infections, further supporting the less sensitive nature of this enzyme compared to CPK and LDH.

Mean AST values of the experimental crocodiles on Day 0 were comparable with normal reference ranges reported for Nile crocodiles (Botha 2010; Lovely *et al*. 2007). Peak AST concentrations did not appear to coincide with the arrival of newborn larvae in muscles but instead reached peak values much later during the course of infection, which coincided with the response observed in LDH. In contrast to ALT, the response of individual crocodiles in the different groups was also more uniform and this suggests that this enzyme is more specific in its response to the parasitic effects of *T. zimbabwensis* than ALT, but less sensitive than CPK and LDH. This is in agreement with reports of increased AST concentrations associated with muscle injuries (Srivastava & Chosdol 2007). Normally, AST levels are higher than ALT and the ratio of AST:ALT are reported to be > 1 (Srivastava & Chosdol 2007). Normal values of these enzymes, as recorded for the experimental crocodiles in this study, support this; AST values were on average higher (31.9 IU/L) than ALT (28.7 IU/L). Srivastava and Chosdol (2007) also reported that AST:ALT ratios may change to ≤ 1 in cases where tissue damage is severe. This was also supported by the results of this study, as mean ALT concentrations (38.8 IU/L) exceeded those of AST (31.8 IU/L) over the course of the experiment.

No reference ranges for CPK for Nile crocodiles have been reported to date. However, mean CPK values of animals in this study on Day 0 were comparable with the ranges reported for American alligators and Dwarf caimans (*Paleosuchus palpebrosus*) (Diethelm & Stein 2006).

The single peak increase in CPK concentrations in the low-infection group on Day 35 PI may correspond with the arrival of larvae and subsequent invasion of muscle tissue. It further suggests that the newborn larvae reached their respective sites of predilection simultaneously, which resulted in a singular, large-scale myopathy event. Analyses of the individual results confirmed that all five animals in this group reached peak CPK concentrations on Day 35 PI. In the medium-infection and high-infection groups, the increase in CPK concentration was not only less pronounced but the subsequent decrease was more protracted compared with the low-infection group. The CPK values remained higher than those recorded on Day 0 in both these groups. This suggests that in these groups, larvae were released into the bloodstream in waves rather than in a single event. This

further supports the hypothesis that development of some larvae may have been delayed. In rats and humans, increases in CPK levels were attributed rather to the level of individual response of the host than to specific damage caused by the parasite (Wisniewska 1970). In this study, however, peak concentrations corresponded with the arrival of newborn larvae in the muscles around Day 35 PI. According to Srivastava and Chosdol (2007), CPK levels may rise within 3–6 h in humans following a heart attack and will return to normal levels within 12–48 h, if no further damage occurs. The results from this study appear to support this, as in the low-infection group, CPK levels decreased immediately after an initial peak on Day 35 PI. However, in the other two groups, CPK did not reach normal levels. This would suggest that muscle damage continued beyond the peak observed on Day 35 PI and further supports the hypothesis that larvae may have been released in waves in the medium-infection and high-infection groups.

Contrary to the case in human trichinosis, increases in CPK levels in this study did not exceed the concurrent increase of other enzymes (Wisniewska 1970) and in fact was less prominent compared with LDH and AST in this study. The fact that CPK levels in the low-infection group did not increase from their lowest levels on Day 49 PI and Day 56 PI, or persist at concentrations higher than those observed on Day 0, may suggest that all of the larvae had invaded the host musculature earlier. No new events of myopathy occurred after Day 35 PI, thus, CPK concentrations decreased to normal levels. However, in the medium-infection group, there is a possibility that the initial damage was more severe and, thus, the decrease in CPK levels was more protracted. In the high-infection group, however, initial damage caused by the larvae was more severe and after Day 35 PI, new larvae continued to be released from the intestine. This explains the subsequent increase in CPK at Day 49 PI. The negative correlation observed between mean blood glucose and mean CPK in the medium-infection group may be explained by the fact that CPK levels continued to decrease beyond Day 49 PI, following its initial peak on Day 35 PI, whereas blood glucose levels increased again after Day 49 PI. If a second wave of larvae was released in this group, as suggested by the rise in glucose levels on Day 49 PI, their numbers may have been too few to cause a significant increase in the already elevated CPK levels recorded on Day 49 PI. In the high-infection group, however, the second wave of larvae resulted in a secondary increase in CPK levels, which coincided with the increase in blood glucose that resulted in the positive correlation observed in this group.

No reference ranges for LDH could be found for Nile crocodiles. Mean LDH of the experimental crocodiles on Day 0 fell well within normal reference ranges reported for Dwarf caimans, as reported by Diethelm and Stein (2006).

In horses infected with *T. spiralis*, increases in CPK, LDH and aldolase all peaked at the fifth week PI and LDH was found to be more sensitive than CPK and aldolase (Soule *et al*. 1989). The sensitivity of LDH was also supported by results from the current study, even though both LDH and AST concentrations peaked later than in the case of CPK. The delayed increase in LDH and AST observed in this study appeared not to coincide with larval migration, but serum concentrations of enzymes might have been influenced by other factors (Srivastava & Chosdol 2007). Although the mean increase in LDH values appeared to be correlated with infection intensity, no direct correlation between these two factors could be established. Koudela and Schanzel (1980) reported that the relationship between levels of LDH and infection intensity was not always apparent when comparing individual animals of the same group. In guinea pigs infected with *Trichinella* spp. larvae, LDH continued to increase until Day 42 PI (Koudela & Schanzel 1980), which was similar to the results observed in the low-infection group in the current study. The continued increase in LDH observed in the medium-infection and high-infection groups once again suggests that larval development and subsequently larval migration may have been delayed in these groups. Similar to CPK, LDH was also negatively correlated with serum glucose in the medium-infection group. Although LDH concentrations increased beyond Day 35 PI, there was also a sharp decrease immediately following a peak on Day 49 PI. The differences in time to reach peak LDH and AST values observed between the experimental groups suggest that these enzymes are not necessarily indicators of muscle damage. The kinetics of these enzymes appears to correspond better with the migration of larvae rather than with actual damage associated with the larvae, this is in agreement with the hypothesis of Koudela and Schanzel (1980).

An indirect ELISA was developed to detect the immune response of crocodiles to *T. zimbabwensis* infection (Ludovisi *et al*. 2013). Immune responses could be detected up to six weeks PI, but not beyond this time, and the ELISA was deemed to be unsuitable for surveillance purposes (Ludovisi *et al*. 2013). Previous studies have shown that the intensity of infection cannot always be correlated with antibody titres (Ludovisi *et al*. 2013; Pozio *et al*. 2002; Vu Thi *et al*. 2010). In crocodiles, interpretation of antibody titres is also complicated, as the immune response may also be influenced by age, temperature, season, and hormone levels (Brown *et al*. 2001; Ludovisi *et al*. 2013). Gamble *et al*. (1996) reported on the efficacy of enzyme immunoassays to detect light infections and indicated that the time period between infection and sero-conversion of the host was problematic in surveillance. The results from the present study confirm this, since the time from infection to sero-conversion ranged from Day 21 PI to Day 56 PI. Kinetics of the antibody response additionally suggest mean sero-conversion in the high-infection group to have been delayed by a week compared to the medium-infection group and by two weeks compared to the low-infection group. Furthermore, in this study, OD values for 10 of the crocodiles decreased in the week following sero-conversion before increasing again. Thus, even if the initial period of infection is known or suspected, surveillance studies will not be effective where they have to rely on a single sample, as the potential will always exist for false negative results to be obtained. Similar to results obtained from a previous study (Ludovisi *et al*. 2013), no

positive correlation between infection intensity and the time to sero-conversion could be established in this study, as has been reported in previous studies involving mammalian hosts (Gottstein *et al*. 2009). Results from this study were also in agreement with those from previous studies where no direct correlation could be established between infection intensity in muscles and antibody levels (Ludovisi *et al*. 2013; Pozio *et al*. 2002; Vu Thi *et al*. 2010).

In crocodiles, the potential effects of individual hormonal status, temperature fluctuations and age on the immune response (Brown *et al*. 2001; Ludovisi *et al*. 2013) may explain these phenomena. However, despite the observed differences between infection intensity and time to sero-conversion, the lower humoral response associated with higher infection levels may also be the result of larvae being released at different intervals, which could result in smaller but repetitive responses from the host. The results revealed similar problems to those reported in mammals infected with other *Trichinella* species, which showed that in most cases antibody titres could not be detected indefinitely (Gottstein *et al*. 2009; Ludovisi *et al*. 2013; Soule *et al*. 1989).

Conclusion

For logistical reasons the study design did not make provision for a control group. However, the results remain valuable, as additional new information about biochemical parameters and humoral immune response of Nile crocodiles (*Crocodylus niloticus*) to infection with *Trichinella zimbabwensis* was obtained.

The fact that hypoglycaemia was not observed in crocodiles experimentally infected with *T. zimbabwensis* suggests that Nile crocodiles may up-regulate blood glucose as the demand for energy is increased by the parasites. This phenomenon requires further investigation in order to elucidate the specific mechanisms and pathways involved, and to determine whether this is common among other ectothermic hosts. Future research should be aimed at determining the influence of infection dose on the rate of development and release of larvae, and the impact of these factors on the different haematological and biochemical parameters of the host.

High variability in ALT changes between individual crocodiles supports the finding that increased ALT activity is not reliable as an indicator for muscle damage and that increases in ALT are rarely observed in cases where damage to the liver is not involved. With exception of the high-infection group, it is suggested that differences in ALT concentrations observed in the crocodiles can largely be attributed to variations in individual physiology of the crocodiles.

The more reliable indicator of muscle damage was CPK, as its peak values correspond with the arrival of larvae in the muscle, in spite of previous reports to the contrary. There was lack of correlation between infection intensity and changes in biochemical parameters and this supports the findings that other host factors, such as hormone levels and the age of the animal, as well as environmental factors, including temperature and season, may impact on the immune response of reptiles. The lack of information on normal reference ranges for haematological and biochemical parameters in Nile crocodiles should be addressed in future research.

Competing interests

The authors declare that they have no financial or personal relationship(s) which may have inappropriately influenced them in writing this article.

Authors' contributions

L.J.L.G. (Department of Agriculture, Rural Development and Land Administration) contributed to the study design and field experiments, data analysis, interpretation and drafting of manuscript and tables. S.M. (University of KwaZulu-Natal) also contributed to the study design and supervision of experiments, assisted with interpretation, drafting and editing of manuscript.

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