UNIVERSITY OF KWAZULU-NATAL

Influence of *Trichinella zimbabwensis* infection intensity on predilection sites, blood biochemical values and humoral immune response in experimentally infected Nile crocodiles (*Crocodylus niloticus*)

By

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DECLARATION

I, Louis Jacobus La Grange declare that

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“A day spent without learning is a day wasted for only one thing cannot be taken from you in this life- KNOWLEDGE”-

Louis J. La Grange Snr.

(1948-2009)

Abstract

The zoonotic potential of *Trichinella zimbabwensis* as supported by the clinical symptoms observed in experimentally infected, non-human primates (Mukaratirwa *et al*., 2001) necessitates research aimed at elucidating the distribution and epidemiology of this parasite.

No controlled studies have been conducted to determine the predilection muscles of *Trichinella zimbabwensis* larvae in Nile crocodiles (*Crocodylus niloticus*) or the influence of infection intensity on the distribution of the larvae in crocodiles. Neither has the influence of *Trichinella zimbabwensis* on biochemical parameters in crocodiles been assessed previously. To determine the distribution patterns of *Trichinella zimbabwensis* larvae and predilection muscles and to assess the influence on selected biochemical parameters, fifteen crocodiles were randomly divided into three cohorts of five animals each to represent high infection (642 larvae/kg of body weight), medium infection (414 larvae/kg of bodyweight) and low infection (134 larvae/kg of bodyweight) cohorts. In the high infection cohort, high percentages of larvae were observed in the tricep muscles (26%) and hind limb muscles (13%). In the medium infection cohort, high percentages of larvae were found in the tricep muscles (50%), sternomastoid (18%) and hind limb muscles (13%). For the low infection cohort, larvae were mainly found in the intercostal muscles (36%), longissimus complex (27%), forelimb muscles (20%), and hind limb muscles (10%). Predilection muscles in the high and medium infection cohorts were similar to those reported in naturally infected crocodiles despite changes in infection intensity. The high infection cohort had significantly higher numbers of larvae in the intercostal, longissimus complex, external tibial flexor, longissimus caudalis and caudal femoral muscles ($P < 0.05$) compared to the medium infection cohort. In comparison to the low infection cohort, the high infection cohort harboured significantly higher numbers of larvae in all muscles ($P < 0.05$) except for the tongue and pterygoid. The high infection cohort harboured significantly higher numbers of larvae ($P < 0.05$) in the sternomastoid, tricep, intercostal, longissimus complex, external tibial flexor, longissimus caudalis and caudal femoral muscles compared to naturally infected crocodiles. The importance of host characteristics in determining predilection and the importance of leg musculature as a predilection site for *Trichinella* spp. in sylvatic carnivores were both confirmed in this study.

Deviations from normal parameters of blood glucose, alanine transaminase (ALT), aspartate transaminase (AST), creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) compared to observations in uninfected reptiles were observed.

Hypoglycaemia was not observed in the infected groups in this study. The humoral immune response to *Trichinella zimbabwensis* infection was evaluated in all three groups by way of indirect ELISA. Peak values of blood glucose, LDH and AST were observed on day 56, 49 and 42 p.i. in the high, medium and low infection cohorts respectively. CPK values peaked on day 35 p.i. in all three cohorts. Peak ALT values were reached on day 56 in the high infection cohort and on day 28 p.i. in both the medium and low infection cohorts. No correlations between the biochemical parameters and infection intensity were observed. Peak antibody titres were reached on day 49 p.i. in the medium infection cohort and on day 42 p.i. in both the high and low infection cohorts. Infection intensity could not be correlated with the magnitude of the humoral immune response or time to seroconversion. The effect of infection intensity on time to seroconversion, magnitude and persistence of the humoral immune response was assessed. No significant differences in the titre levels between the three groups were observed. Infection intensity could not be correlated with the magnitude of the humoral response or time to seroconversion. Results of this study were in agreement with results reported in mammals (wild boars and horses) infected with other *Trichinella* species and showed that antibody titres could not be detected indefinitely.

List of contents

		Page
Declaration		ii
Acknowledgements		iii
Abstract		v
List of contents		vii
List of Tables, Figures and Plates		ix
Addendum- Ethical clearance		
Chapter 1.	Introduction and Literature Review	
	1.1 Introduction.	1
	1.2 Literature Review.	6
	1.2.1 Characteristics and classification of *Trichinella zimbabwensis*.	6
	1.2.2 Distribution of *Trichinella zimbabwensis*.	7
	1.2.3 Epidemiology.	8
	1.2.4 Diagnosis, treatment and control.	9
	1.2.5 Factors precluding the efficacy of control and prevention of infection with *Trichinella zimbabwensis*.	11
	1.3 References.	14
Chapter 2.	General Methodology	
	2.1 Source of study animals.	23
	2.2 Aspects of animal husbandry.	23
	2.2.1 Housing of experimental animals.	23
	2.2.2 Feeding of experimental animals.	24
	2.3 References	24
Chapter 3.	Assessment of distribution patterns and predilection muscles of *Trichinella zimbabwensis* larvae in experimentally infected Nile crocodiles (*Crocodylus niloticus*).	26
	3.1 Abstract	26
	3.2 Introduction	27
	3.3 Materials and Methods	28
	3.3.1 Source and preparation of infective material	28

3.3.2 Infection of experimental animals 28
3.3.3 Collection of muscle samples 29
3.3.3.1 Biopsy samples 29
3.3.3.2 Euthanasia of infected animals and post mortem sampling 30
3.3.4 Testing of muscle samples 31
3.3.4.1 Artificial Digestion 31
3.4 Data Analysis 32
3.5 Results 33
3.6 Discussion 35
3.7 Conclusion 38
3.8 References 39
Chapter 4. Assessment of selected biochemical parameters and humoral immune response of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*. 47
4.1 Abstract 47
4.2 Introduction 48
4.3 Materials and Methods 50
4.3.1 Source and preparation of infective material 50
4.3.2 Infection of experimental animals 50
4.3.3 Collection of serological samples 51
4.3.3.1 Preservation of samples 52
4.3.4 Testing of blood glucose and serological samples 52
4.3.4.1 Glucose testing 52
4.3.4.2 Testing for antibody titres 52
4.3.4.3 Testing of enzyme levels 53
4.4 Data Analysis 53
4.5 Results 54
4.5.1 Blood Glucose 54
4.5.2 Alanine transaminase (ALT) 54
4.5.3 Aspartate transaminase (AST) 55
4.5.4 Creatine phosphokinase (CPK) 55
4.5.5 Lactate dehydrogenase (LDH) 56

4.5.6 Indirect ELISA 57
4.6 Discussion 58
4.6.1 Influence of infection intensity on Blood glucose 58
4.6.2 Influence of infection on alanine transaminase (ALT) 60
4.6.3 Influence of infection on aspartate transaminase (AST) 60
4.6.4 Influence of infection intensity on creatine phosphokinase (CPK) 61
4.6.5 Influence of infection intensity on lactate dehydrogenase (LDH) 63
4.6.6 Influence of infection intensity on humoral immune response 64
4.7 Conclusion 65
4.8 References 66
Chapter 5. General Discussion and Conclusion 83
5.1 General Discussion 83
5.2 General Conclusion 86
5.3 References 87

Page

List of Tables, Figures and Plates

Tables

Table 3.1 Reproductive Capacity Index (RCI) of *Trichinella zimbabwensis* larvae in experimentally infected Nile crocodiles (*Crocodylus niloticus*) 60 days post infection. 43
Table 3.2 Mean (lpg/muscle) distribution of *Trichinella zimbabwensis* in individual muscles of experimentally and naturally infected crocodiles (*Crocodylus niloticus*). 44
Table 3.3 Mean (lpg/muscle) distribution of *Trichinella zimbabwensis* larvae in grouped muscles of experimentally and naturally infected Nile crocodiles (*Crocodylus niloticus*). 45
Table 3.4 Comparison of distribution of *Trichinella zimbabwensis* larvae in tail musculature of Nile crocodiles (*Crocodylus niloticus*) samples through necropsy and biopsy. 46

Table 4.1 Haematological reference ranges of blood glucose and enzyme levels in a variety of crocodilian species. 76
Table 4.2 Mean glucose concentration in sera of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*. 77
Table 4.3 Mean alanine transaminase (ALT) concentration in sera of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*. 78
Table 4.4 Mean aspartate transaminase (AST) concentration in sera of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*. 79
Table 4.5 Mean creatine phosphokinase (CPK) concentration in sera of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*. 80
Table 4.6 Mean lactate dehydrogenase (LDH) concentration in sera of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*. 81
Table 4.7 Mean change in anti- *Trichinella* lgG in sera of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*. 82

Figures

Figure 1.1 Natural distribution of Nile crocodiles (*Crocodylus niloticus*) and known distribution of *Trichinella zimbabwensis* in Africa. 20
Figure 1.2 Hypothetical sylvatic cycle of *Trichinella zimbabwensis* in Africa. 21
Figure 2.1 Architectural drawing of crocodile enclosures used to house experimental animals. 25
Figure 4.1 Mean glucose increase in Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*. 71
Figure 4.2 Percentage change in alanine transaminase (AST) concentration in Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*. 72
Figure 4.3 Percentage change in creatine phosphokinase (CPK) concentration in Nile crocodiles (*Crocodylus niloticus*)

experimentally infected with *Trichinella zimbabwensis*. 73

Figure 4.4 Percentage change in lactate dehydrogenase (LDH) concentration in Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*. 74

Figure 4.5 Mean changes in anti- *Trichinella* lgG in Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*. 75

Plates

Plate 1.1 Adult male Nile crocodile (*Crocodylus niloticus*) on a farm in South Africa. 8

Plate 1.2 A skinned and eviscerated carcass of a Nile crocodile (*Crocodylus niloticus*). 12

Plate 2.1 Oral infection of a Nile crocodile (*Crocodylus niloticus*) with *Trichinella zimbabwensis* larvae. 22

Plate 3.1 Biopsy sampling from the dorso-lateral aspect of the tail in a Nile crocodile (*Crocodylus niloticus*). 30

Plate 3.2 Post mortem sampling of crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis* larvae. 31

Plate 3.3 Microscopic view of *Trichinella sp*. L1 larvae. 32

Plate 4.1 Collection of blood from the supra-vertebral sinus in a Nile crocodile (*Crocodylus niloticus*) 51

Chapter 1

Introduction and Literature Review

1.1 Introduction

The genus *Trichinella* is composed of eight species (*T. spiralis*, *T. nativa*, *T. britovi*, *T. pseudospiralis*, *T. murrelli*, *T. nelsoni*, *T. papuae*, *T. zimbabwensis*) and four additional genotypes (*Trichinella* T6 related to *T. nativa*; T8 and T9 related to *T. britovi* and *Trichinella* T12) (Pozio & Zarlenga, 2005; Pozio *et al*., 2009). The genus belongs to the family Trichinellidae and the Order Trichurida, phylum Nematoda (Pozio *et al*., 2009). Two unique characteristics separate the *Trichinella* genus from other nematodes and the first one is that the host fulfils the requirements of both definitive and intermediate stages in the parasite life cycle and the second one is that the first stage larvae (L1) represents the infective stage of the parasite rather than the third stage larvae (L3) as is commonly found in the majority of nematodes (Pozio, 2007, Pozio *et al*., 2009). The adult nematodes are found in the intestines whilst the larvae reside in skeletal muscle fibres.

With the exception of Antarctica, species/genotypes in the genus are widespread throughout the world (Pozio & Murrell 2006; Pozio *et al*., 2009; Mukaratirwa *et al*., 2012). In nature these parasites infect a large variety of sylvatic carnivores and omnivores (Pozio, 2005; Pozio, 2007; Pozio *et al*., 2009). Nematodes in the genus *Trichinella* have, until recently, only been detected in warm blooded animals. The five encapsulated species (*T. spiralis*, *T. nativa*, *T. britovi*, *T. murrelli* and *T. nelsoni*) as well as the two non-encapsulated species (*T. pseudospiralis* and *T. papuae*) have only been detected in mammalian species except for occurrences of *T. pseudospiralis* in birds (Pozio, 2007). In 1999, *T. papuae* was found to be capable of completing its life cycle in experimentally infected reptiles (Pozio *et al*., 2004) and natural infection has been reported in saltwater crocodiles (*Crocodylus porosus*) and in wild pigs (*Sus scrofa*) in Papuae New Guinea (Pozio *et al*., 2005). Another non-encapsulated species, *T. zimbabwensis,* was first detected in crocodiles in Zimbabwe and proved capable of infecting reptiles and mammals (Mukaratirwa and Foggin 1999; Pozio *et al*., 2002; Mukaratirwa *et al*., 2008).

Apart from their sylvatic animal hosts several *Trichinella* spp. infect humans and the most important species are *T. spiralis* and *T. britovi* (Gottstein *et al*., 2009). Humans become infected through the consumption of raw or undercooked meat from infected

animals (Dupoy-Camet, 2000, Gottstein *et al*., 2009). The zoonotic importance of *Trichinella* forms the basis for the implementation of control measures aimed at the control or eradication of the parasite from the human food chain (Gottstein *et al*., 2009; Mukaratirwa *et al.*, 2012). However, despite implementation of control measures these parasites remain a major zoonosis threat in many parts of the world (Murrell & Pozio 2011, Mukaratirwa *et al*., 2012). Many reasons have been cited to explain the failure of control measures in developed countries, most notably the cosmopolitan distribution of members of the *Trichinella* genus (Pozio, 2007; Mukaratirwa *et al*., 2012), cultural eating habits that favour the transmission of the parasite (Dupoy-Camet, 2000; Pozio, 2007; Mukaratirwa *et al*., 2012), poor animal husbandry (Pozio, 2000; Pozio, 2001), globalization (Dupoy-Camet, 2000), changing political environments that resulted in reduced veterinary controls (Dupoy-Camet, 2000; Pozio, 2001; Gottstein *et al*., 2009), misdiagnosis of the disease due to physicians' unfamiliarity with its clinical manifestations (Dupoy-Camet, 2000; Gottstein *et al*., 2009), ecological changes (Dupoy-Camet, 2000) including the establishment of Trans Frontier Conservation Areas (TFCA's) (Mukaratirwa *et al*., 2012) and a lack of proper communication and reporting between countries (Dupoy-Camet, 2000).

The adoption of biotechnology have improved the diagnosis, identification and reporting of outbreaks that may additionally explain the emergence of new infection patterns (Pozio, 2001; Pozio & Zarlenga, 2005; Pozio & Murrell, 2006; Pozio *et al*., 2009). Nevertheless, the improved ability to identify species under the genus is of limited value if the access to their natural hosts is limited. Pozio (2005) reported that many epidemiologically important host species are protected by national and international legislation which complicates access to these species. Since sylvatic, carnivorous and omnivorous animals are known hosts of *Trichinella* spp., surveillance among these species is crucial. However, physical capture of these animals is often not feasible and costly. Additionally, the collection of sufficient volumes of sample through biopsy is difficult. In order to ensure detection of *Trichinella* infection, direct methods of testing such as the artificial digestion method requires a high level of sensitivity, especially in animal hosts since they do not present readily observable clinical manifestations (Gottstein *et al*., 2009). The accuracy of direct testing methods is dependent on the sample size, sampling site and the application of the correct method of testing (Gottstein *et al*., 2009). Through the evaluation of predilection muscles in different hosts, recommendations on suitable sampling sites, sample size and preferable methods for detection of larvae have been developed (Gottstein *et al*., 2009).

Establishing general consensus on the best sampling sites for detection of *Trichinella* is not easily achieved as several factors influence predilection patterns of the different species in their respective hosts. According to a study conducted on *T. spiralis* and *T. britovi* infection in pigs, animals with a low infection intensity harbour more larvae in the base of the tongue than in the diaphragm muscles whereas in heavier infections it was found that the muscle predilection pattern changed with significantly higher numbers of larvae in diaphragm muscles than in the base of the tongue (Serrano & Pérez-Martín, 1999). According to Wright *et al*. (1989), the distribution of larvae in light infections may be attributed to the passive transportation of larvae in the bloodstream and that larvae will only survive if they establish themselves in myofibres surrounded by venous capillary networks.

The supply of blood to individual muscle groups is usually correlated with the frequency and intensity of movement required from that muscle (Folkow and Halicka, 1968; Andersen and Henriksson, 1977). Frequently used muscles have a higher blood supply in order to maintain the metabolic processes of those muscles (Folkow and Halicka, 1968; Andersen and Henriksson, 1977). Reina *et al*. (1996) reported that the most active muscles usually harbour the most larvae. Kapel *et al*. (1995) hypothesized that larval burden is more dependent on the muscle's potential to move rather than the actual frequency of movement as determined by the host's level of activity. This was demonstrated when Arctic foxes (*Alopex lagopus*) kept in cages (Kapel *et al*., 1994) exhibited similar patterns of infection in their muscles to those observed in free-living foxes despite the obvious restrictions imposed on the movement of the leg musculature of caged animals (Kapel, 1995). Wright *et al*. (1989) reported a difference between the development sites of *T. spiralis* and *T. pseudospiralis* where the newborn larvae of the former only develop in slow twitch fibres and those of the latter will develop in both slow and fast twitch fibres.

A study in monkeys revealed that adults and larvae of these two species in particular show differences in their ability to survive the host immune response with adults of *T. pseudospiralis* being more vulnerable in the intestinal phase and resistant in the muscle phase than those of *T. spiralis* (Kociecka *et al*., 1980). A study by Kapel *et al*. (2005) showed a significant difference in muscle predilection between non-encapsulated species and encapsulated species in foxes. Interestingly, a study by Hurníková *et al*. (2004) involving Red foxes (*Vulpes vulpes*) infected with *T. zimbabwensis* also showed different patterns in muscle predilection from those observed in the study by Kapel *et al*. (2005) which involved foxes and *T. pseudospiralis*. However, predilection patterns in Red foxes and Arctic foxes infected

with *T. spiralis* and *T. nativa* respectively, were reported to be similar (Kapel *et al*., 2005). The aforementioned studies show that differences between the *Trichinella* taxa also influence muscle predilection.

The geographic distribution and to a certain extent, species specificity of different *Trichinella* taxa suggest that some environmental factors also influence the ability of the parasite to infect potential hosts. Most notably, temperature tolerance of the different taxa is a significant determinant for both geographic distribution and infectivity. Pozio *et al*. (2009) summarized the infectivity of different *Trichinella* species according to the temperature ranges preferred by their respective hosts. Climatological factors also directly impact on the survival of these parasites and the association between infectivity, geographic distribution, freezing tolerance and survival in decaying flesh have been discussed previously (Hurníková *et al*., 2004; Pozio *et al*., 2009). Important as the above factors may be, results from several studies have suggested host characteristics to be the most important determinant for predilection (Soule *et al*., 1989; Kapel, 1995; Reina *et al*., 1996; La Grange *et al*., 2013). Kapel *et al*. (1995) summarised the predilection characteristics from previous studies on herbivorous, carnivorous and omnivorous hosts and concluded that differences exist between carnivorous and herbivorous hosts.

Several studies on mammals have been conducted to find alternative methods for the detection of *Trichinella* infection. The efficacy of serological testing has been evaluated and includes enzyme immunoassay tests conducted on horses (Soule *et al*., 1989; Gamble *et al*., 1996), indirect immunofluorescence assays in horses (Soule *et al*., 1989) and ELISA techniques in horses and goats (Soule *et al*., 1989; Reina *et al*., 1996). However, specific antibodies against *Trichinella* could only be detected for a short period of time following infection. In horses, IgG assayed by ELISA could only be detected from two weeks post infection (p.i.) and was only detectable until the 27th week p.i. whereas immunofluorescence techniques detected IgG only until 23 weeks p.i. (Soule *et al*., 1989). Gamble *et al*. (1996) also reported on the efficacy of enzyme immunoassays to detect even light infections but indicated that the time period between infection and sero-conversion of the host was problematic in surveillance. In a study involving rats infected with *T. spiralis*, expulsion of adult worms only started between days eight to ten p.i. and in some cases continued until 28 days p.i. (Love *et al*., 1976). Once ingested, larvae mature in the host intestine within 36-48 hours and develop into adults within four to five days (Fabre *et al*., 2009). This short developmental period does not allow the host to launch an effective immune response against the adult worms until they have reproduced (Fabre *et al*., 2009) which explains

the delay in sero-conversion and the subsequent effective establishment of newborn larvae (NBL). Furthermore, larvae and adults are antigenically heterogenous, which further preclude an effective humoral response (Fabre *et al*., 2009). Little information is available concerning the antibody response of the host against muscle stages of the parasite but a mixed isotype response of IgG1, IgG2 and IgE have been reported in chronic infections with IgG1 being the most dominant (Fabre *et al*., 2009). Immunoassays have been reported to be a "potential substitute for artificial digestion methods" but the study animals were all euthanized at 12 weeks p.i. (Gamble *et al*., 1996) and 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). The disappearance of antibody titres over time renders these serological tests obsolete for surveillance studies because animals that have been infected for extended periods of time will not be identified. The only exception to this rule was reported in pigs where antibody titres persisted for longer periods of time and are presumed to remain detectable indefinitely except in cases where wild boars are less susceptible to certain *Trichinella* species (Gottstein *et al*., 2009).

Several studies have reported deviations in specific enzyme levels from normal biochemical parameters where *Trichinella* infection is concerned (Dusanic, 1966; Tassi *et al*., 1995; Ribicich *et al*., 2007). Serum levels of creatine phosphokinase (CPK), lactate dehydrogenase (LDH), and aspartate transaminase (AST) are used in diagnostic procedures to detect *Trichinella* infections in humans (Gottstein *et al*., 2009). Increased serum levels of alanine transaminase (ALT) in pigs infected with *T. spiralis* have also been reported previously (Ribicich *et al*., 2007).

Reports of human trichinellosis in sub-Sahara African countries have, in comparison to the rest of the world been very rare (Mukaratirwa *et al*., 2012). According to Pozio *et al*. (2005) customary practices in food preparation and religion, especially those religious laws that forbid pork consumption, can be correlated with the rare incidence of human disease on the African continent. This may at first glance appear to negate the need to commit valuable time and expensive resources to research aimed at the control of this parasite. However, Bengis and Veary (1997) expressed concern that the potential for human infection may actually be raised through other cultural practices such as traditional healing. This author also noted that some local cultures in South Africa believe that the consumption of meat from various carnivorous animals, including lions can confer specific benefits to the consumer and, depending on the species of animal consumed, may provide strength and longevity.

This study involved two distinct objectives. The first objective was aimed at elucidating the predilection muscles and distribution patterns of *T. zimbabwensis* larvae in Nile crocodiles experimentally infected with high, medium and low doses of first stage larvae (L1). Knowledge of the predilection patterns may be useful to improve current sampling methods and provide consensus on the disparate views concerning the appropriate sampling sites. The second objective was to assess the relationship between infection intensity and selected biochemical parameters as well as the antibody response of Nile crocodiles to experimental infection with *T. zimbabwensis* to investigate the suitability of serological methods to detect *T. zimbabwensis* in crocodiles.

1.2 Literature Review

1.2.1 Characteristics and classification of *T. zimbabwensis*

Trichinella zimbabwensis is classified as a non-encapsulating *Trichinella* species based on the absence of a collagen capsule surrounding the larva in host musculature (Pozio *et al*., 2002; Pozio & Zarlenga 2005; Pozio *et al*., 2009). Adult males average 1066µm and females 1096µm in length (Pozio *et al*., 2002). Experimental studies with *T. zimbabwensis* in mammals and reptiles showed larvae of this parasite specie to be larger in poikilothermic hosts than in mammalian hosts and is probably due to the host metabolic rate (Pozio *et al*., 2004). The adult and larval stages of *T. zimbabwensis* are morphologically similar to those of *T. papuae*. Cross breeding of adult males and females of both species have been observed, although the F1 offspring only produce few and less viable F2 larvae. DNA comparisons between *T. zimbabwensis*, *T. papuae* and *T. pseudospiralis* also confirmed that larvae of *T. zimbabwensis* are more related to these two non-encapsulated species than to any of the encapsulated species (Pozio *et al*., 2002).

Trichinella adults and larvae are intracellular parasites (Despommier, 1993). Adults reside within the mucosa of the intestinal wall while larvae parasitize striated muscle cells (Gottstein *et al*., 2009). *Trichinella* establish in the host when larvae contained in raw or undercooked meat is consumed (Dupoy-Camet, 2000). The larvae are released following digestion of the meat during normal gastric processes (Gottstein *et al.*, 2009). The larvae rapidly moult four times and develop into adults and newborn larvae are released within five to seven days following the initial period of infection (Gottstein *et al*., 2009). The first stage larvae (F1) are transported by the circulatory system to the predilection sites where they penetrate muscle cells and can potentially survive for

many years (Bruschi, 2012). The larvae of *T. zimbabwensis* are not resistant to freezing and were found not to be infective after 10 days at -10°C (Pozio *et al*., 2002). Studies involving mice revealed that the parasite can, in mammals, be transmitted from mother to offspring (Mukaratirwa *et al*., 2001) and both congenital and transmammary infection routes were confirmed in rats (Matenga *et al*., 2006). Molecular investigation through multiplex PCR showed that these parasites display a 264 bp band unique to this species (Pozio *et al*., 2009) but that isolates form different geographical regions may be heterogenous (Pozio *et al*., 2009; La Grange *et al*., 2009). Studies in experimentally infected varans (*Varanus niloticus*) and caimans (*Caiman crocodilus*) reported no clinical manifestations of disease and support the hypothesis that these animals serve as natural hosts of the parasite (Pozio *et al*., 2004). Experimental studies in baboons and monkeys reported clinical disease symptoms similar to those observed in human infections with other *Trichinella* spp. supporting the zoonotic potential of *T. zimbabwensis* (Mukaratirwa *et al*., 2001).

1.2.2 Distribution of *T. zimbabwensis*

In 1995, *Trichinella* infection was found in farmed crocodiles (*Crocodylus niloticus*) in Zimbabwe with a prevalence of 40% (Foggin *et al*., 1997). The parasite was described as a new species and named *T. zimbabwensis* and was experimentally found to be infective to reptiles and mammals, including non-human primates (Mukaratirwa & Foggin 1999; Pozio *et al*., 2002; Mukaratirwa *et al*., 2008). Subsequent surveys also indicated the prevalence of *T. zimbabwensis* in wild crocodiles (*C. niloticus*) in Lake Cahora Bassa, Mozambique, and Ethiopia as well as monitor lizards (*Varanus niloticus*) in Zimbabwe (Mukaratirwa and Foggin, 1999; Pozio *et al*., 2002; 2007).

During the period 1985 – 1987, several farmers imported breeding stock from Zimbabwe and Botswana to help establish crocodile farming in South Africa (La Grange *et al*., 2009). Although *T. zimbabwensis* was only described in Zimbabwe in 1995, the initial period of infection in Zimbabwe is unknown. According to records obtained from the National Department of Agriculture, approximately 30700 crocodiles were also imported into South Africa from Mozambique over a four year period from 2002 up until the implementation of the import ban on 14 November 2005. Recent surveys in South Africa reported a high prevalence of *T. zimbabwensis* in wild crocodile populations in the Kruger National Park (La Grange *et al*., 2009; La Grange *et al*., 2013). The first reported naturally infected mammal was a lion (*Panthera leo*) (La Grange *et al*., 2010) in South Africa. The reports however represent only a small number of animals from four African countries and the presence of the parasite in

neighbouring countries (Mukaratirwa *et al*., 2012) as well as other countries where Nile crocodiles are known to exist still requires investigation.

Plate 1.1 Adult male Nile crocodile (*Crocodylus niloticus*) on a farm in South Africa



A large number of breeding stock such as this male photographed on a farm in South Africa were originally imported from neighbouring countries during the 1980's to help establish crocodile farming in South Africa. The crocodile showed here originated from Botswana.

Nile crocodiles are the second largest of the 23 known crocodilians (Huchzermeyer, 2003), and the most widespread species of crocodiles in Africa (Botha, 2010). These reptiles naturally inhabit 43 African countries including the island of Madagascar (Botha 2010). Magnino *et al.* (2009) reported that Nile crocodiles are farmed in several African countries (Kenya, Zimbabwe, Tanzania and South Africa) as well as in Israel, Indonesia, France, Japan and Spain and that licensing for farming was issued in the UK in 2006. Thus an urgent need exists for epidemiological surveys in the remaining 39 countries encompassing the natural range of Nile crocodiles and additional surveillance and control in the other countries since current information on the distribution of *T. zimbabwensis* is limited. The natural distribution of Nile crocodiles in Africa and the known distribution of *T. zimbabwensis* are shown in Figure 1.1.

1.2.3 Epidemiology

Several experimental studies have been conducted in mammalian and reptilian hosts and have reported on the developmental stages of *T. zimbabwensis* (Pozio *et al*., 2002;

Hurníková *et al*., 2004; Pozio *et al*., 2004; Matenga *et al*., 2006; Mukaratirwa *et al*., 2008). However, the natural epidemiology of the parasite is yet to be fully elucidated. Based on the current knowledge of natural reservoirs it is hypothesized that the parasite's natural life cycle is largely maintained through predatory, cannibalistic and scavenger behaviour of crocodiles towards members of its own species and varans as well as scavenger behaviour of varans towards crocodiles (Pozio *et al*., 2007, La Grange *et al*., 2009; Mukaratirwa *et al*., 2012). The discovery of a naturally infected mammal in South Africa has opened up a new avenue of exploration into unravelling the epidemiology of this species (La Grange *et al*., 2010) and suggests that other carnivorous and omnivorous mammals may be involved in the natural epidemiology of this species (Mukaratirwa *et al*., 2012). Mukaratirwa *et al*. (2012) proposed a hypothetical sylvatic cycle that includes both known and potential sylvatic reservoirs of *T. zimbabwensis* (Figure 1.2). The infection on commercial farms in Zimbabwe was maintained through feeding of infected carcasses to other crocodiles on the farm (Pozio *et al*., 2005) but as far as a domestic cycle is concerned, the maintenance of infection through potential synantrophic hosts on commercial crocodile breeding farms has not been investigated.

1.2.4 Diagnosis, treatment and control

No human infections with *T. zimbabwensis* have been reported to date and there is little information on the distribution and epidemiology of this parasite. More than 65 000 cases of trichinosis in humans, including 42 fatalities were confirmed between 1986 and 2009 (Murrell & Pozio, 2011; Mukaratirwa *et al*., 2012). Despite differences in the biological and molecular structure of the species, clinical manifestations of the disease in humans follow a specific pattern with varying intensity dependent on the infection dose and species of *Trichinella* involved (Kociecka, 2000). Symptoms vary in accordance with the stage of the parasite and include those associated with gastro-intestinal disease during the enteral phase of the parasite in the gut as well as muscular myositis in the systemic phase (Gottstein *et al*., 2009). The disease may manifest itself as an acute or chronic infection but patients can remain asymptomatic depending on the initial infection dose (Gottstein *et al*., 2009).

Diagnostic protocols for the detection of human infection have been well described and rely on the assessment of clinical symptoms, laboratory findings and epidemiological investigation (Gottstein *et al*. 2009). Following a positive diagnosis, treatment regimes include anthelmintics including albendazole, mebendazole, pyrantel (Kociecka, 2000;

Gottstein *et al*., 2009) or thiabendazole (Kociecka, 2000). Glucocorticosteroids and protein and electrolyte replacement preparations (Kociecka, 2000; Gottstein *et al*., 2009) as well as immunomodulating drugs (Kociecka, 2000) should also be included.

Diagnosis in animals relies on the detection of muscle larvae through direct testing methods such as trichinoscopy and artificial digestion (European Commission, 2005) whilst identification at the species level requires molecular techniques (Gottstein *et al*., 2009). Current testing protocols for export of crocodile meat in South Africa require that samples be collected from the anterior legs of the slaughtered animals. This is considered as one of the predilection muscles of *T. zimbabwensis.* Despite the suitability of the anterior leg musculature for testing, European Commission Regulation 2075/2005 (2005) recommends sampling from the masseter, pterygoid or intercostal muscles. An indirect ELISA was developed to detect the humoral immune response of crocodiles to *T. zimbabwensis* but was found to be unsuitable for surveillance purposes due to the unpersistence of antibodies (Ludovisi *et al.,* 2013). Results from naturally infected crocodiles did suggest some potential for the use of biopsy sampling to aid in surveillance (La Grange *et al*., 2009; 2013) but since neither the initial infection levels nor the influence thereof on predilection have been studied, the reliability of these methods remain questionable. Studies in baboons and monkeys showed treatment with ivermectin to be effective (Mukaratirwa *et al*., 2008). However, treatment of crocodiles has not been attempted.

The relatively low number of deaths (6.38%) reported for the period 1986-2009 (Mukaratirwa *et al*., 2012) may suggest effective treatment for humans. However, the cost of treatment exceeds that of preventative control measures (Gottstein *et al*., 2009). Prevention of human infections is only possible if the potential transmission from both sylvatic and domestic hosts is adequately controlled. Transmission from domestic hosts should theoretically be easier to prevent since domestic animals often are bred, slaughtered and processed in a controlled commercial environment. The success of any preventative measures however depends on the proper implementation thereof in all stages of the production and thus requires a “farm–to-fork” approach. Sound animal husbandry practises on farm level, especially proper disposal of animal carcasses are crucial to prevent transmission of the parasite between crocodiles and from crocodiles to other domestic animals. Veterinary control through testing and/or treatment of meat products is essential. Treatment options aimed at inactivating or killing the parasite in meat include cooking (> 71°C core temperature), freezing (-15°C for three to four weeks) and irradiation (0.3kGy) (Gottstein *et al*., 2009). Proper cooking as described above and sourcing meat from reputable sources such as approved slaughterhouses

are key measures that the end consumers of meat can implement to protect themselves.

1.2.5 Factors precluding the efficacy of control and prevention of infection with *T. zimbabwensis*

Despite effective diagnostic and treatment regimes for human infections, the disease has the potential to go unnoticed among many people on the African continent where the risk of infection is considerable. The potential existence and recurrence of human infections must not be underestimated and the lack of information on human infections should in actual fact form the basis for proper control measures to prevent occurrence until the perceived negligible risk can be disproved on the basis of sufficient scientific research.

In South Africa the majority of game farms are situated in rural areas that are often remote and lack infrastructure (Bengis and Veary, 1997). This is certainly also true for most other wildlife reserves on the African continent. The remote and extensive nature of game reserves prevents the establishment of proper slaughter and processing infrastructure, meat inspection and access to specialized veterinary services and tests to detect infected meat before consumption (Bengis and Veary, 1997). Many resource poor communities are dependent on local populations of wildlife as a source of food and often share other basic natural resources such as water with potential sylvatic hosts of *Trichinella*. One such example is the co-existence of Nile crocodiles with fisherman on the shores of Lake Kariba (McGregor, 2005). According to Gottstein *et al*. (2009) a key factor in the prevention of human infection involves the education of consumers in the potential risk of the disease. Providing basic education to the resource-poor communities in rural Africa may be a considerable challenge. Not only does their remote and often inaccessible locality cause logistical difficulty, but many of these communities have deeply rooted cultural beliefs and practises that may not agree with preventative strategies, especially those concerning proper food preparation.

In Africa, certain cultures may prefer the services of traditional healers rather than those of conventional physicians and in some cases individuals may be forced by their socio-economic status to rely on these cheaper alternative medicines that could lead to underreporting of the disease. Misdiagnosis of the disease by physicians may additionally hamper its detection and control (Dupoy-Camet, 2000).

In sylvatic animals several hurdles hamper the effective detection of *T. zimbabwensis*. The musculature at the base of the tongue is one of those favoured by *Trichinella spp.* in other hosts (Reina *et al*., 1996). The tongue is generally not used for diagnostic purposes in crocodiles because it is covered by a superficial layer that is indigestible and not easily removed, preventing the detection of larvae. The general digestibility of the tongue musculature is also lower than that of many other muscles resulting in longer digestion times (Kapel *et al*., 2005). Bearing in mind the zoonotic potential of *Trichinella* spp. it is important that the predilection patterns of *T. zimbabwensis* in the crocodile musculature be determined. Currently the procedures for sampling and testing of crocodiles contained in the EU Regulations are similar to those described for other wild animals (European Commission, 2005). Specific regulations for crocodiles are not foreseen in the near future since crocodile meat is not a product of any of the EU member countries. The lack of approved methods for sampling in live animals is also problematic for surveillance. Furthermore, testing of crocodile meat destined for local markets is not required in South Africa which leaves consumers at risk of infection.

Plate 1.2 A skinned and eviscerated carcass of a Nile crocodile (*Crocodylus niloticus*)



Meat derived from carcasses such as this, destined for local markets in South Africa, are not subjected to testing for *Trichinella.*

The parasite is also of economic importance to small scale crocodile producers in South Africa due to the high costs incurred in testing the meat for *Trichinella*. Recent

reports indicate that the cost of testing is becoming a burden for the larger, export approved facilities (Pfitzer and Huchzermeyer, *unpublished*).

Because *T. zimbabwensis* is a non-encapsulated species, it would be reasonable to expect the host immune response to be stronger and more persistent due to the direct contact between the parasite larvae and host tissue (Huchzermeyer, personal communication, 2008). An experimental study to determine the efficacy of ELISA for the detection of *T. zimbabwensis* infection in crocodiles was conducted (Ludovisi *et al*., 2013). However, the results revealed similar problems to those reported in mammals infected with other *Trichinella* species and showed that in most cases, antibody titres could not be detected after six weeks p.i. Dzik (2006) reviewed the different methods employed by helminth parasites to evade the immune response and reported several molecules released by *T. spiralis* as a strategy to evade the immune system of host. In the case of reptiles, other host factors such as hormone levels and the age of the animal as well as environmental factors including temperature and season, may also impact on the immune response (Brown *et al*., 2001; Ludovisi *et al*., 2013).

Normal biochemical values for several crocodile species have been reported (Millan & Janmaat, 1997; Stacy & Whitaker, 2000; Lovely *et al*., 2007; Padilla *et al*., 2011) but to the author's knowledge no studies have been conducted to compare the effect of infection on the normal biochemical parameters of crocodiles. A study by Wisniewska (1970) showed that changes in CPK levels observed in rats infected with *T. spiralis* may harbour some diagnostic potential although the evaluation of CPK alone cannot be considered "a specific test for trichinosis."

Apart from the limitations of diagnostic tools, specific factors surrounding the natural hosts, and in particular crocodiles, additionally exacerbate the problem. All of the known crocodile species are listed in either Appendix I or Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [Cites, 2012].

Despite the fact that crocodiles are known to be natural hosts of *T. zimbabwensis* (Pozio, 2005; Pozio *et al*, 2007; La Grange *et al*., 2009), to date most experimental studies have focussed on the parasite's relationship with mammalian hosts (Hurníková *et al*., 2004; Matenga *et al*., 2006; Mukaratirwa *et al*., 2008;. Host characteristics are known to be important determining factors of the parasite-host relationship and especially predilection (Soule *et al*., 1989; Kapel, 1995; Reina *et al*., 1996; La Grange *et al*., 2013). Thus, the differences between poikilothermic and homeothermic host species provide the incentive for controlled studies aimed at elucidating the specific

interactions between *T. zimbabwensis* and its natural hosts which are of considerable importance to develop specific, accurate control measures to prevent human infection.

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Figure 1.1. Natural distribution of Nile crocodiles (*Crocodylus niloticus*) and known distribution of *Trichinella zimbabwensis* in Africa.

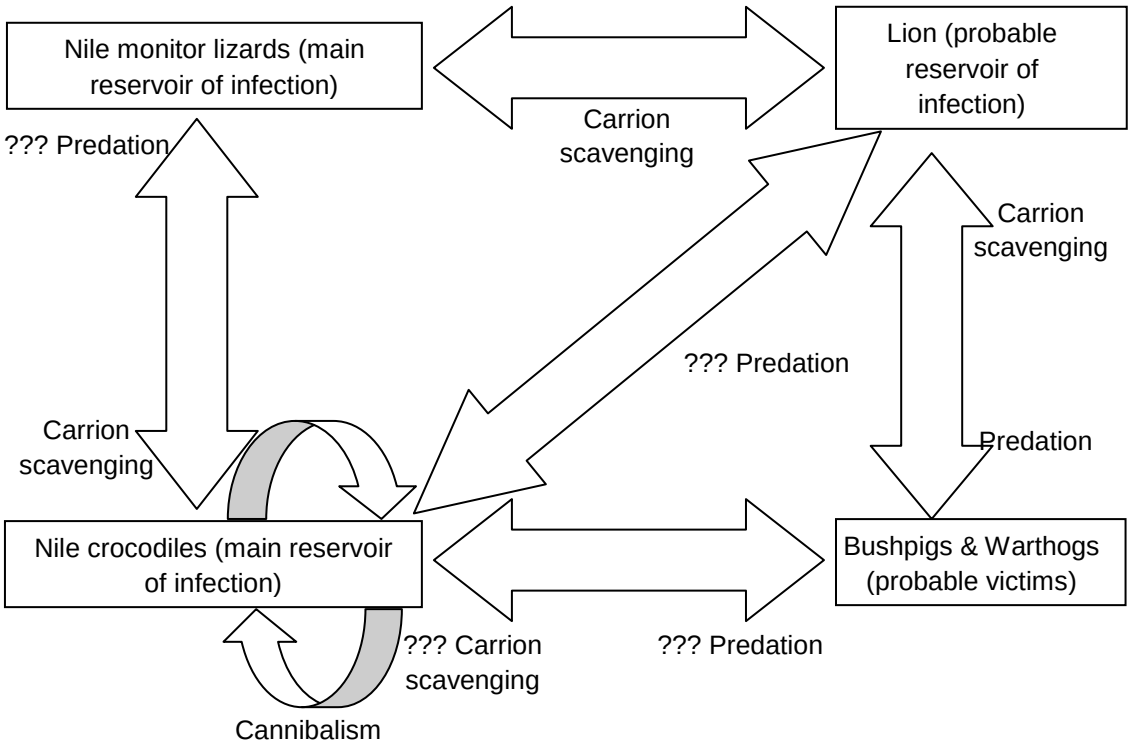


*Distribution of Nile crocodiles extrapolated from Botha (2010).

**Distribution of *T. zimbabwensis* extrapolated from Pozio *et al*. (2007) and La Grange *et al*. (2013).

Map of Africa extrapolated from http://d-maps.com/carte.php?lib=africa_map&num_car=4339&lang=en

Figure 1.2 Hypothetical sylvatic cycle of *Trichinella zimbabwensis* in Africa



* Extrapolated from Mukaratirwa *et al*. (2012).

Chapter 2

General Methodology

To determine the distribution patterns of *Trichinella zimbabwensis* larvae and predilection muscles and to assess the influence on selected biochemical parameters, fifteen crocodiles were randomly divided into three cohorts of five animals each to represent high infection (642 larvae/kg of body weight), medium infection (414 larvae/kg of bodyweight) and low infection (134 larvae/kg of bodyweight) cohorts.

Plate 2.1 Oral infection of a Nile crocodile (*Crocodylus niloticus*) with *Trichinella zimbabwensis* larvae.



2.1 Source of study animals

Fifteen seven-year old crocodiles (13 males and 2 females) within the size range of 1.35 – 1.80 metres in length were used. The crocodiles represented a group of runts sourced from a commercially farmed population with no history of *T. zimbabwensis* infection prior to commencement of the study. The runting was not caused by any underlying health problem and was attributed to individual physiological and/ or genetic factors which caused these animals to grow at a slower rate compared to other individuals of similar age (Huchzermeyer, 2003). The use of runts for the purposes of this study was financially motivated as their reduced commercial value made them more accessible. The animals were captured on the Wilderness Roads farm in Low's Creek, Mpumalanga Province and immobilized with 0.4ml of gallamine triethiode (40mg/ml) (Kyron) injected intramuscularly on the lateral aspect of the tail base of each animal before being transported to the experimental housing.

The crocodiles were randomly divided into the respective cohorts prior to the capturing of pre-trial data. This strategy ensured that any conscious or subconscious bias towards specific animals based on their size, sex or any other observable physical characteristics was removed. Pre-trial data collected from the animals include weight, sex and length of the animals. Each animal was marked by means of scute clipping for easy identification. For the high infection cohort both the left and right horizontal tail scutes were clipped in sequence according to the number assigned to the animal. For the low and medium infection cohorts only the scutes on the left or right were clipped respectively. The experiment was carried out from January- March 2012, when climatic conditions ensured good feeding and optimal physical condition of the animals.

2.2 Aspects of animal husbandry

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

2.2.1 Housing of experimental animals

The study animals were housed on a smallholding belonging to the Mpumalanga Tourism and Parks Agency (MTPA) on the outskirts of Nelspruit, Mpumalanga. A fenced enclosure (10 x 5m) was constructed using 65mm diamond mesh fencing with a single access gate. A concrete pond was constructed in the middle of the enclosure measuring 7 x 3m. The pond was sloped and ranged in depth from 600mm -1.2m. This allowed for a temperature gradient in the water to provide for optimal thermoregulation.

A shaded area was also provided in one corner of the enclosure using 80% shade netting. The area surrounding the pond was covered with grass to reduce the risk of injury to both the animals and personnel during weekly capture and sampling (Figure 2.1). The pond was drained on a weekly basis and replenished with fresh water during capture and sampling. In addition chlorine was added to the water (40 ppm) using small chlorine floaters similar to those used for swimming pools to prevent the excessive growth of algae and bacteria in the water.

2.2.2 Feeding of experimental animals

Animals were fed with coarsely minced chicken carcasses. To optimise feeding and reduce stress, food was enriched by the addition of vitamins and minerals. The vitamin and mineral content of the minced chicken was enriched with a specially formulated vitamin premix additive marketed commercially for crocodiles (Feedmix, Johannesburg). The premix consists of separate mineral and vitamin components that are supplemented as 2.5kg and 1.25kg respectively in a 1 000kg of wet ration. Due to the smaller volumes of feed required for the experimental animals, enriched food was sourced as pre-packed rations from Seronera crocodile farm in Hazyview, Mpumalanga. The commercial premix does not contain calcium and this was separately supplemented with calcium powder ($CaCo_3$) (Kyron) added to the food to a final composition of 1.5% of the total food ration.

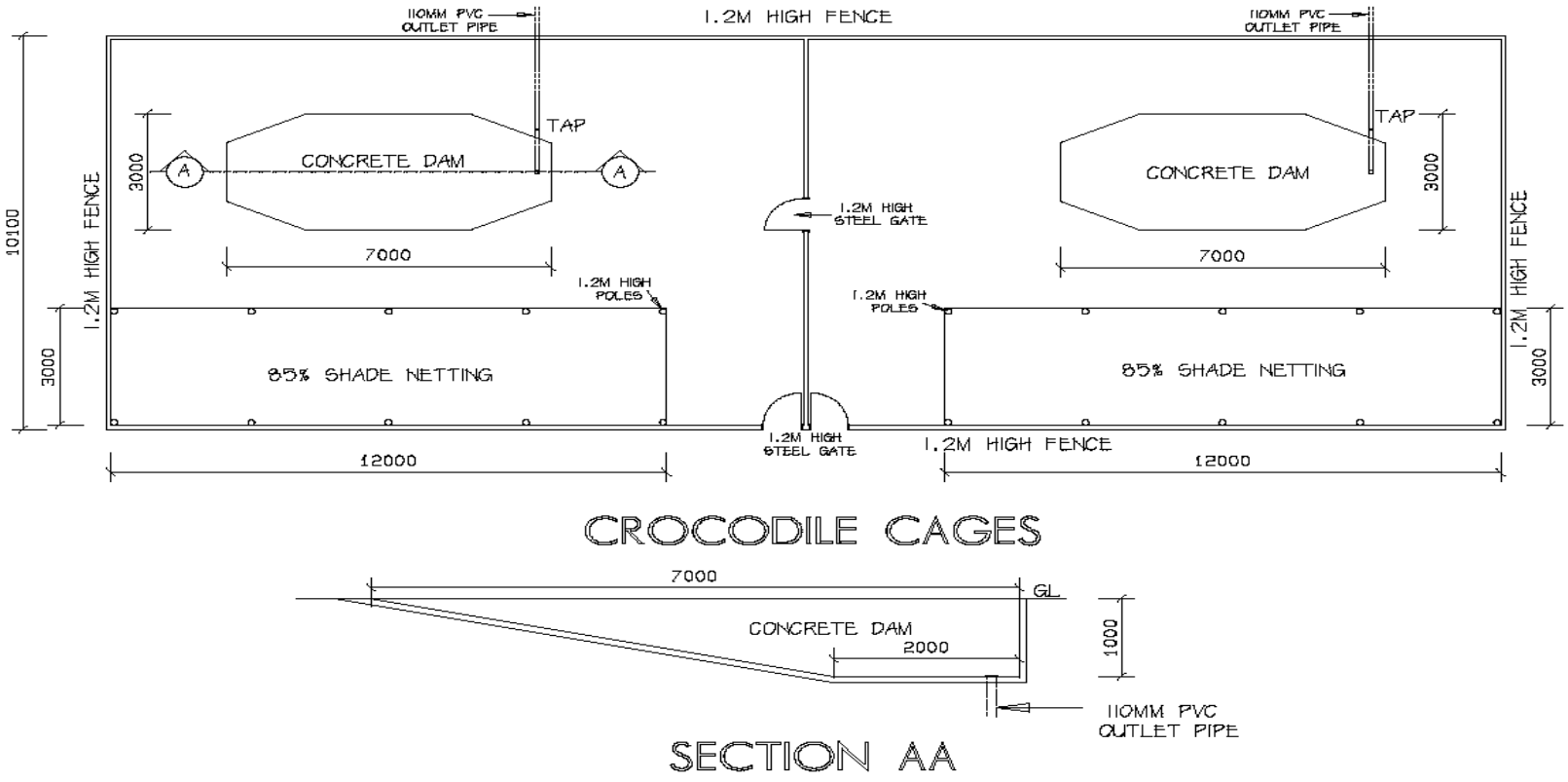
Crocodiles were not fed individually but approximately 10 kilograms of food was offered two to three times a week depending on climatic conditions. During cool and rainy spells food was offered less frequently as the crocodiles ate less when cooler weather resulted in decreases in their metabolic rate. Any leftover food was removed from the enclosures to maintain good hygiene. No food was offered on days immediately prior to testing to ensure that blood glucose levels were not influenced by the intake of food.

2.3 References

Huchzermeyer, F.W., 2003. Crocodiles: Biology, husbandry and diseases. 1st edition, CABI Publishing, ISBN 0-85199-656-6.

SABS Standards Division, 2009. South African National Standard: Crocodiles in captivity. SANS 631:2009, 1st Edition, ISBN 978-0-626-22294-9.

Figure 2.1 Architectural drawing of crocodile enclosures used to house experimental animals.



Architectural drawing drafted by J. Steyn, Nelspruit, Mpumalanga.

Chapter 3

Assessment of distribution patterns and predilection muscles of *Trichinella zimbabwensis* larvae in experimentally infected Nile crocodiles (*Crocodylus niloticus*)

3.1 Abstract

No controlled studies have been conducted to determine the predilection muscles of *Trichinella zimbabwensis* larvae in Nile crocodiles (*Crocodylus niloticus*) or the influence of infection intensity on the distribution of the larvae in crocodiles. However, the European Commission Regulation 2075/2005 recommends sampling from the masseter, pterygoid and intercostal muscles for detection of larvae in crocodiles. The distribution of larvae in several muscles of naturally infected crocodiles *(C. niloticus)* and experimentally infected caimans (*Caiman crocodilus*) and varans (*Varanus exanthematicus*) have been reported in literature. In order to determine the distribution patterns of *T. zimbabwensis* larvae and predilection muscles, fifteen crocodiles were randomly divided into three cohorts of five animals each to represent high infection (642 larvae/kg of body weight), medium infection (414 larvae/kg of bodyweight) and low infection (134 larvae/kg of bodyweight) cohorts. In the high infection cohort high percentages of larvae were observed in the tricep muscles (26%) and hind limb muscles (13%). In the medium infection cohort high percentages of larvae were found in the tricep muscles (50%), sternomastoid (18%) and hind limb muscles (13%). For the low infection cohort larvae were mainly found in the intercostal muscles (36%), longissimus complex (27%), forelimb muscles (20%), and hind limb muscles (10%). The predilection muscles in the high and medium infection cohorts were similar to those reported in naturally infected crocodiles despite changes in infection intensity. The high infection cohort had significantly higher numbers of larvae in the intercostal ($P < 0.05$), longissimus complex ($P < 0.05$), external tibial flexor, longissimus caudalis and caudal femoral muscles ($P < 0.05$) in comparison with the medium infection cohort. In comparison to the low infection cohort, the high infection cohort harboured significantly higher numbers of larvae in all of the muscles ($P < 0.05$) with exception of the tongue and pterygoid. Compared to naturally infected crocodiles, the high infection cohort also harboured significantly higher numbers of larvae ($P < 0.05$) in the sternomastoid, tricep, intercostal, longissimus complex, external tibial flexor, longissimus caudalis and caudal femoral muscles.

The larvae of *T. zimbabwensis* favoured the musculature of the abdominal region including the muscles of the anterior and posterior limbs and appear first to invade predilection muscles closest to their release site in the small intestine before occupying those muscles situated further away. The tricep was the most important predilection site in the high infection and medium infection cohorts. The importance of host characteristics in determining predilection and leg musculature as a predilection site for *Trichinella* spp. in sylvatic carnivores, were both confirmed in this study. Results from this study also support the use of biopsy sampling from the dorso-lateral regions of the tail for surveillance purposes.

3.2 Introduction

Knowing the predilection muscles of *Trichinella* spp. larvae in hosts is important in order to improve the detection of the parasite in animal hosts especially with low levels of infection (Kapel *et al*., 2005). Artificial digestion is the only diagnostic method currently approved for the detection of *Trichinella* spp. in sylvatic animals (European Commission, 2005). Gottstein *et al*. (2009) indicated that the sensitivity of the test is important as animal hosts do not develop observable clinical symptoms and factors such as sample size and sampling site influence the test sensitivity.

Several studies have been conducted to determine predilection sites of different *Trichinella* spp. in various hosts (Kociecka *et al*., 1980; Reina *et al*., 1996; Serrano & Pérez-Martín, 1999, Kapel *et al*., 1994, 1995, 2005). These studies have led to recommendations on the types of muscles to sample, the quantity and the appropriate method(s) for detection in several animal hosts (Gottstein *et al*., 2009). However, knowledge of predilection sites alone is not sufficient to improve detection.

Studies have shown that the predilection sites of *Trichinella* spp. is influenced by several factors including differences between species (Wright *et al*., 1989; Kapel *et al*., 2005), initial levels of infection (Serrano & Pérez-Martín, 1999) and host characteristics (Soule *et al*., 1989; Kapel *et al*., 1995; Reina *et al*., 1996; Hurníková *et al*., 2004; Kapel *et al*., 2005). No controlled studies have been conducted to determine predilection muscles in Nile crocodiles or the influence of infection intensity on the distribution of *T. zimbabwensis* larvae in crocodiles. The distribution of larvae in several muscles of naturally infected crocodiles has been reported previously (La Grange *et al*., 2013) and in experimentally infected caimans (*Caiman crocodilus*) and varans (*Varanus exanthematicus*) (Pozio *et al*., 2004).

Current testing protocols for the detection of *Trichinella* larvae in muscles for export of crocodile meat in South Africa require that samples be collected from the anterior limbs of the slaughtered animals in contrast to the European Commission Regulation 2075/2005 which recommends sampling from the masseter, pterygoid or intercostal muscles in crocodiles.

The objective of this study was to determine the influence of infection intensity on distribution patterns and predilection sites of *T. zimbabwensis* larvae in experimentally infected Nile crocodiles.

3.3 Materials and Methods

To determine the distribution patterns of *Trichinella zimbabwensis* larvae and predilection muscles, fifteen crocodiles were randomly divided into three cohorts of five animals each to represent high infection (642 larvae/kg of body weight), medium infection (414 larvae/kg of bodyweight) and low infection (134 larvae/kg of bodyweight) cohorts.

3.3.1 Source and preparation of infective material

Infective material was sourced from a crocodile experimentally infected with *T. zimbabwensis*. Muscle tissue was collected from various sites, minced and thoroughly mixed by hand (in protective clothing) using a ladle to stir the minced material to a homogenised sample. 100 Grams of the homogenised sample was processed by artificial digestion and infection level was determined to be 30 larvae per gram (LPG) of the homogenized sample. Infective material for each individual animal was separately packaged and refrigerated at 4°C until the day prior to infection.

3.3.2 Infection of experimental animals

Infective material was removed from the refrigerator the evening before infection to allow it to reach room temperature.

In order to allow adequate time to conduct euthanasia of the experimental animals and testing of samples for each cohort of animals, the cohorts were infected at weekly intervals.

The animals were starved for at least 72 hours prior to infection to facilitate the infection process and prevent regurgitation of infective material caused by overfilled stomachs.

In order to administer the infective material, each animal was restrained by hand, the eyes and mouths taped shut and the animal placed on a table and secured by hand. The tape around the mouth was removed and a perspex tube 20mm long with a diameter of 40mm was placed in the tip of animal's mouth and taped into position to facilitate the insertion of the stomach tube and to ensure that the tube is not crushed. A thin dowel stick was used to open the gular valve at the back of the mouth and pressed flat against the mouth floor to allow insertion of a stomach tube. A stomach tube (700mm in length, 20mm in diameter) was inserted through the opening of the perspex tube into the oesophagus and carefully pushed into the opening of the stomach.

Once the tube was in the stomach, the animal was held in a vertical position and a short stem funnel was attached to the stomach tube and the required amount of infective material was trickled down into the tube and pushed towards the stomach with a dowel stick. Once all of the infective material was administered, a small amount of water was used to wash down any material that may have been caught on the sides of the tube. The animal was then placed on the ground and the tape and perspex tube removed. The animals were continuously monitored for at least thirty minutes following infection to ensure that they do not regurgitate.

3.3.3 Collection of muscle samples

3.3.3.1 Biopsy sampling

Muscle biopsies were collected from the dorso-lateral aspects of the tail base on day 28 post infection (p.i.) in all cohorts. Prior to biopsy, a muscle relaxant, gallamine triethiodide, 40mg/ml (Kyron) was administered intramuscularly at a dose of 0.4ml to each animal. A local anaesthetic, lignocaine (Kyron) was administered around the biopsy site. An incision was made with a scalpel through the skin between the rows of scutes and extended laterally across the length of two scutes near the tail base. The incision was also extended ventrally across the length of one scute to form a square angle with the first incision (Plate 3.1). This created a flap of skin which was later closed again over the wound. A minimum of 10 grams of muscle tissue was removed with a scalpel and forceps taking care not to collect tissue from the deeper

musculature. The samples were refrigerated at 4°C until testing. The biopsy wound was closed by pressing the flap of skin back into its original position over the wound and held in place with sutures. The remaining edges of the skin was bonded with a quick drying, gel based, waterproof adhesive and 0.1 MIU/kg penicillin administered intramuscularly (Huchzermeyer, 2003) to prevent wound infections.

Plate 3.1 Biopsy sampling from the dorso-lateral aspect of the tail in a Nile crocodile (*Crocodylus niloticus*).



3.3.3.2 Euthanasia of infected animals and post mortem sampling

Animals from each cohort were euthanised on day 60 p.i. following procedures outlined by Beaver *et al*., 2001 (Plate 3.2).

Carcasses were skinned and samples collected from the base of the tongue, external pterygoid, sternomastoid, triceps brachii, longissimus complex, intercostal pillars, longissimus caudalis and caudal femoral muscles/ muscle group of each animal using diagrams of the musculature as presented by Richardson *et al*. (2002) to ensure collection from the correct muscles. Approximately 50 grams of muscle tissue were collected from each of the muscles/muscle groups. In addition to the above, 10 gram samples were collected from the superficial, lateral aspects of the longissimus complex and illoischiodalis muscles of tail to mimic biopsy samples in live animals. All of the samples were placed in leak proof containers and refrigerated at 4°C until tested.

Plate 3.2 Post mortem sampling of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis* larvae.



3.3.4 Testing of muscle samples

3.3.4.1 Artificial digestion

Pooled samples representing individual animals were prepared by collecting 10 grams of muscle tissue from all of the individual muscles (tongue, pterygoid, sternomastoid, tricep, intercostal, longissimus complex, external tibial flexor, longissimus caudalis and caudal femoral*) and combining them in a single sample of 90 grams. For individual muscle digestion, 25 grams of tissue was used from each muscle and 10 grams of muscle tissue were used to mimic biopsies.

Samples were artificially digested according to Nöckler & Kapel (2007).

**The use of the term "illoischiodalis" muscle as reported by La Grange et al. (2013) is in error, the correct terminology for this muscle is caudal femoral as referred to in this study.*

Plate 3.3 Microscopic view of *Trichinella* sp. L1 larva.



Light microscope x 1000 magnification

3.4 Data analysis

Data obtained from naturally infected crocodiles (La Grange *et al*. 2013) were included in the analyses and compared with data from this study. Data was normalized [$\log_{10}(x+1)$] and one of the naturally infected animals which had an unusually high level of infection compared to other naturally infected animals reported by La Grange *et al*. (2013) was removed from the analysis.

Muscles were grouped together to represent the cranial, abdominal and caudal muscle regions of crocodiles. The cranial group of muscles included the tongue, pterygoid and sternomastoid. The abdominal muscle group consisted of the tricep, intercostals, longissimus complex and tibial flexor muscles. The caudal muscle group was represented by the longissimus caudalis and caudal femoral muscles including the biopsy samples. The mean infection intensity of the muscles in each of these three regions was calculated for each infection cohort and expressed as a percentage of the combined mean of all three regions.

Initial dosage/kg body mass was correlated with overall infection intensity in each cohort using Spearman's rho correlation analyses (IBM SPSS Statistics 19).

Reproductive capacity indices (RCI) were calculated for larvae in each crocodile (Oivanen *et al*., 2002) and expressed as a mean value per infection cohort.

The mean larval burdens were compared using analysis of variance (IBM SPSS Statistics 19) to determine any significant differences within the muscles of each cohort. A t- test was used to compare larval burdens between the superficial- and deep musculature of the tail and between the dorsal- and ventral biopsy samples. Significance level was set at $P < 0.05$.

3.5 Results

The mean dose of infection (larvae/kg body weight) and the mean reproductive capacity index (RCI) for each cohort are shown in Table 3.1. Mean RCI was 8.06, 0.89 and 0.13 for the high, medium and low infection cohorts respectively. Results of the correlation analyses showed the infection dose to be negatively correlated with overall infection intensity ($R^2 = -1.000$, $P < 0.001$) and RCI in the high infection cohort ($R^2 = -0.825$, $P < 0.05$).

The mean larvae per gram of muscle sample (lpg) in pooled samples for the high, medium and low infection cohorts was 5.18, 0.37 and 0.02 respectively (Table 3.2). The lpg in pooled samples was comparable with that from naturally infected crocodiles although lower than that of the high infection cohort but higher than that of the medium and low infection cohorts of this study. A high mean lpg was also observed in all of the individual muscles of the high infection cohort when compared to natural infections. The mean lpg was also greater in the sternomastoid, tricep, longissimus complex and external tibial flexor muscles of the medium infection cohort when compared to the natural infections.

In the low infection cohort, muscle larvae established in very low numbers (< 0.04 lpg) in all the 5 individuals and in one animal only a single larvae was detected in the pool sample. The highest percentage of larvae in the other four remaining animals was found in the intercostal (36%), longissimus complex (27%), forelimb (20%), and hind limb (10%) regions with the highest percentage of larvae occupying the intercostal- and longissimus complex muscles. In the medium infection cohort all of the muscle regions tested was infected but the higher percentage of larvae were found in the tricep muscles (50%) followed by the sternomastoid (18%) and hind limbs (13%). A more even distribution of larvae was noted in the high infection cohort with tricep muscles

(26%) and hind limbs (13%) harbouring higher numbers of larvae. In naturally infected crocodiles the tricep muscles harboured 17% of all larvae with most of the remaining larvae spread more evenly among the pterygoid (12%), superficial longissimus caudalis (12%), external tibial flexor (10%), intercostal (10%), illoischiodalis (10%) and deep longissimus caudalis (9%). The medium infection cohort had significantly higher numbers of larvae in the intercostal, longissimus complex, external tibial flexor, longissimus caudalis and caudal femoral muscles ($P < 0.05$) when compared with the low infection cohort. The high infection cohort harboured significantly higher numbers of larvae in all of the muscles compared to the low infection cohort ($P < 0.05$) with exception of the tongue and pterygoid. The high infection cohort also harboured significantly higher numbers of larvae in the sternomastoid, tricep, intercostal, longissimus complex, external tibial flexor, longissimus caudalis and caudal femoral muscles ($P < 0.05$) compared to the naturally infected crocodiles.

The abdominal muscle groups harboured the highest number of larvae in all of the cohorts and revealed less variation in the distribution of larvae between the caudal and cranial muscle groups (Table 3.3). Significantly higher numbers of larvae ($P < 0.05$) were found in the cranial muscle group of crocodiles in the high infection cohort compared to those of the low infection cohort and naturally infected crocodiles but not when compared to the medium infection cohort. Differences in larval burdens of the cranial muscle groups were not significant ($P > 0.05$) between any of the other cohorts. The high infection cohort also showed significantly higher numbers of larvae ($P < 0.05$) in the abdominal and caudal muscle groups compared to any of the other cohorts with no significant difference among the other cohorts. In the high and medium infection cohorts the abdominal muscle groups harboured 56% and 59% of larvae respectively followed by the cranial (21% and 28%) and caudal (23% and 13%) muscle groups respectively. In the low infection cohort the abdominal muscle group harboured 82% of all larvae with the remaining larvae almost equally spread in the caudal (10%) and cranial (8%) muscle groups. The naturally infected animals showed very little difference in distribution of larvae between the three muscle groups with 38% of larvae found in the abdominal, 33% in the caudal and 29% in the cranial muscle groups.

Artificial digestion and examination of the biopsy samples collected on day 28 post infection (p.i.) showed no larvae in any of the three cohorts. However, biopsy samples collected on day 60 p.i. were positive for *T. zimbabwensis* larvae (Table 3.2). Differences observed in larval distribution between the superficial and deep musculature of the tail were not significant although the dorsal biopsy samples in all the cohorts on average harboured slightly more larvae (1.25 lpg) than the deeper

musculature (0.88 lpg). Conversely, the biopsy samples collected from the ventral muscles harboured a smaller average number of larvae (0.78 lpg) than the deeper musculature (1.21 lpg). Average larval burdens in tail musculature for individual cohorts are shown in Table 3.4.

3.6 Discussion

In this study crocodiles that received a lower number of larvae relative to their bodyweight showed higher infection intensity after 60 days p.i. in the high infection cohort. Similar results were reported by Hurníková *et al*. (2004) in a study involving Red foxes (*V. vulpes*) experimentally infected with *T. zimbabwensis* but the observed difference was not significant. Although the infective dose was not expressed per kilogram bodyweight, the foxes were reported to be adult, farm bred animals which may suggest uniformity in weight. The variation in infection dose/kg body weight in this study may explain the observed significance of the correlation. The negative correlation observed between RCI and the initial infection dose as well as between initial infection dose and LPG in the high infection cohort of this study, suggests that when a specific maximum threshold of infective larvae is exceeded, it triggers a severe reaction from the host immune system to counter the infection. This heightened immune response would result in decreased numbers of infective larvae and fecund adults, subsequently giving rise to fewer newborn larvae (NBL) to invade the host musculature.

This, although it at first glance appears to have a negative influence on the parasite, may ultimately benefit both the host and parasite. If a maximum number of larvae is exceeded and allowed to develop into fecund adults, the subsequent release of large larval burdens in the blood circulation could potentially be fatal to the host as a result of restricted blood flow to vital organs or acute anaphylaxis which would cause the demise of the parasite as well.

The high numbers of larvae observed in the tricep, intercostal and external tibial flexor muscles of the crocodiles do not support the findings observed in experimentally infected caimans (*C. crocodilus*) and varans (*V. exanthematicus*) where the tongue harboured the highest number of larvae (Pozio *et al*., 2004). However, the importance of the muscles of the fore and hind limbs in sylvatic carnivores as reported by Kapel *et al*. (1994, 1995) is in agreement with findings from this study.

It appears that predilection sites of *T. zimbabwensis* in Nile crocodiles is not influenced by the locomotive potential of muscles as seen in foxes (Kapel *et al*., 1994, 1995) since in crocodiles the limbs are rarely associated with the high frequency and intensive locomotive behaviour compared to land based animals. Crocodiles generally travel only short distances on land at slow speed and mainly use their large tail muscles to swim and propel themselves when acquiring prey (Richardson *et al*., 2002). Thus, in crocodiles the notion that the most active muscles are the most parasitized seems not to apply (Reina *et al*., 1996). However, the locomotive behaviour of crocodiles when submerged at the bottom of rivers and lakes has not been studied intensively and their leg musculature may play a more significant role under such circumstances than is currently known.

The results from this study showed the tricep muscle to harbour the most larvae in natural (17%), medium (50%) and high infections (26%). In low infections the intercostal muscles harboured the most larvae (40%). Deviations in predilection patterns between different levels of infection were also noted in similar studies in mammals (Gamble *et al*., 1996; Serrano & Pérez-Martín, 1999).

The results show that larvae primarily establish in those muscles in close proximity of the abdominal muscle region and disperse in relatively equal numbers to the cranial- and caudal muscle regions further away from their initial release site in the small intestine as infection levels are increased. The dispersion of- and subsequent increase in larval numbers in the cranial and caudal muscle regions appear to be correlated with a simultaneous and proportional decrease of larvae in the abdominal muscle region that eventually leads to an approximately equal distribution in all three muscle regions. Individually however the muscles favoured by the larvae as predilection sites retain their proportionally higher numbers of larvae. This is consistent with the hypothesis that larvae of *Trichinella* will primarily seek out predilection muscles in cases of low infection and will invade alternate muscle groups that are available as the infection intensity is increased (Wright *et al*., 1989).

However, this does not explain the relatively more uniform regional distribution of larvae observed in naturally infected crocodiles compared to crocodiles of the high infection cohort. This phenomenon may be the result of secondary and subsequent recurring infections of crocodiles in the wild. Importantly, crocodiles with naturally acquired infections comprised of individuals with large variations in size that were for the most part much larger and therefore much older than those used in the experimental cohorts. The naturally infected crocodiles additionally were derived from

various habitats within the Kruger National Park. It is known that host factors such as hormone levels and the age of the animal as well as environmental factors including temperature and season, may also impact on the immune response of crocodiles (Brown *et al*., 2001; Ludovisi *et al*., 2013). Considering the aforementioned, the potential for variation in individual immunological status of the naturally infected crocodiles could additionally also influence the distribution patterns of larvae in these animals.

In experimental studies involving *T. spiralis* and *T. pseudospiralis* in monkeys, larvae were detected in biopsy samples from day twelve and day 24 respectively (Kocieska *et al*., 1980). In goats infected with *T. spiralis*, muscle larvae were detected from day 20 p.i. (Reina *et al*., 1996) and from day 21 p.i. in heavily infected horses (Soule *et al*., 1989). In this study biopsy samples from all three experimental groups collected on day 28 p.i. tested negative for *T. zimbabwensis* larvae but larvae were detected in biopsy samples collected on day 60 p.i. These differences between crocodiles and mammals cannot at this stage be accurately explained but studies in caimans and varans experimentally infected with *T. zimbabwensis* and *T. papuae* showed that muscle tissue damage occurred more rapidly in mammals than in reptiles and suggested that the host physiology could play a role in this (Pozio *et al*., 2004). Results from this study appear to support this hypothesis especially considering that the metabolic rate of a crocodile weighing 70 kilograms is far lower than that of a human of similar mass (Huchzermeyer, 2003). The study by Pozio *et al*. (2004) additionally showed that larvae of *T. zimbabwensis* had a longer growth period and subsequently developed larger in size in reptilian hosts than in mammals. This longer growth period was hypothesized to cause a delay in larvae establishing in muscle tissue (Pozio *et al*., 2004).

In mammals, the rate of establishment of larvae can also be influenced by infection intensity and larvae tend to establish earlier in high infections than in low infections (Soule *et al*., 1989). Kocieska *et al*. (1980) also demonstrated that the rate of larval establishment varied between different species of *Trichinella.* In experimental studies involving *T. spiralis* and *T. pseudospiralis* in monkeys, larvae were detected in biopsy samples from day 12 p.i. and day 24 p.i. respectively (Kocieska *et al*., 1980). In goats infected with *T. spiralis* muscle larvae were detected from day 20 p.i. (Reina *et al*., 1996) and from day 21 p.i. in heavily infected horses (Soule *et al*., 1989).

T. zimbabwensis might undergo a longer period of growth in reptilian hosts resulting in delayed establishment in the muscles (Pozio *et al*., 2004). However, the impact of a

slower metabolic rate in crocodiles should not be ignored as host physiology may influence the rate at which larvae establish (Pozio *et al*., 2004).

3.7 Conclusion

Trichinella zimbabwensis larvae successfully established in the muscles of all the experimentally infected animals of this study. The results are in agreement with findings in experimentally infected Red foxes (*V. vulpes*) (Hurníková *et al*., 2004) where larger infection doses correspond with a lower RCI of the larvae.

Studies aimed at determining the maximum threshold for infection dose in different size and age groups of crocodiles are required. Additionally it is not clear whether the decrease in parasite numbers is purely a host strategy aimed at survival or whether the parasite itself is in some way responsible for the decrease of its own numbers to ensure preservation of the host.

Despite habitat and physiological differences between crocodiles and land based mammals, results from this study support the importance of leg musculature as a predilection site for *Trichinella* spp. in sylvatic carnivores (Kapel *et al*., 1995). Although crocodiles appear to use their limbs less frequently and intensively compared to land-based mammals, more research is required to elucidate the use of their limbs when submerged at the bottoms of rivers and lakes.

Furthermore, results from this study show that, in Nile crocodiles, larvae of *T. zimbabwensis* appear first to invade predilection muscles closest to their release site in the small intestine before occupying those muscles situated further away. This is in agreement with the hypothesis of Wright *et al*. (1989) that the larvae of *Trichinella* spp. would establish first in predilection muscles before occupying other available muscles. Studies involving additional experimental animals should be conducted to further investigate this hypothesis and to establish the influence of challenge infections on the distribution patterns of these larvae.

In Nile crocodiles, based on this study, the tongue does not appear to be a predilection site for *T. zimbabwensis* larvae as is the case in varans and caimans (Pozio *et al*., 2004). The difference in predilection muscles observed between caimans (Pozio *et al*., 2004) and Nile crocodiles in this study further support the importance of host characteristics as a determinant for predilection (Soule *et al*., 1989; Kapel *et al*., 1995; Reina *et al*., 1996; Hurníková *et al*., 2004; Kapel *et al*., 2005). Additional factors which

may impact the immunological response of crocodiles such as temperature, season, age of the animal and hormone levels (Brown *et al*., 2001; Ludovisi *et al*., 2013) may also have led to variations in larval distribution patterns as observed between caimans (Pozio *et al*., 2004) and crocodiles of this study.

The recommendation for the use of masseter, pterygoid and intercostal muscles as sampling sites for the detection of *T. zimbabwensis* in crocodiles (European Commission, 2005) is in contrast to the results from this study where the fore- and hind limb muscles had the highest number of larvae.

In this study biopsy samples from all three experimental cohorts collected on day 28 p.i. tested negative for *T. zimbabwensis* larvae but subsequently were detected in biopsy samples collected on day 60 p.i.

These results appear to support the hypothesis that larvae of *T. zimbabwensis* might undergo a longer period of growth in reptilian hosts resulting in delayed establishment in the muscles (Pozio *et al*., 2004).

Results from this study additionally support the use of biopsy sampling from the dorso-lateral regions of the tail for surveillance purposes in both wild- and commercial crocodile populations (La Grange *et al*., 2013).

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Table 3.1. Reproductive capacity index (RCI) of *Trichinella zimbabwensis* larvae in experimentally infected Nile crocodiles (*Crocodylus niloticus*) 60 days post infection.

Cohort	N	Mean Weight (kg)/Animal	Mean Dose Larvae/kg BW	Mean LPG	Mean RCI
High Infection	5	15.56	642	5.18	8.06
Medium Infection	5	12.08	414	0.37	0.89
Low Infection	5	14.96	134	0.02	0.13

BW = Body Weight; LPG = Larvae per gram

Table 3.2. Mean (lpg/muscle) distribution of *Trichinella zimbabwensis* in individual muscles of experimentally and naturally infected Nile crocodiles (*Crocodylus niloticus*).

	High infection (N = 5)											
	Pooled	**1**	**2**	**3**	**4†**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Mean (lpg)	5.18	0.56	3.46	6.12	16.24	6.86	4.6	8.56	3.02	5.34	4.32	2.14
SE	2.16	0.15	1.43	1.86	4.77	1.72	1.81	2.28	0.63	1.55	1.08	0.29
Range	1.8 – 13.6	0.2 -1.1	0 - 8	0.3 – 10.9	2.8 – 27.9	2.5 – 11.9	0.4 – 9.2	1.7 - 16	1.7 – 5.3	1.6 – 9.4	2.5 – 8.4	1.5 – 3.2
	Medium infection (N = 5)											
	Pooled	**1**	**2**	**3**	**4†**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Mean (lpg)	0.37	0.06	0.59	1.38	3.92	0.35	0.33	1.03	0.27	0.14	0.44	0.45
SE	2.42	0.02	0.46	1.16	2.47	0.19	0.18	0.50	0.17	0.12	0.14	0.27
Range	0.03 – 1.3	0 - 0.1	0 - 0.3	0 - 2.4	0 - 6	0.08 – 12.4	0 - 0.9	0 - 0.9	0.04 – 2.4	0 - 0.9	0 - 0.6	0.2- 0.9
	Low infection (N = 5)											
	Pooled	**1**	**2**	**3**	**4**	**5†**	**6**	**7**	**8**	**9**	**10**	**11**
Mean (lpg)	0.02	0	0.01	0.01	0.05	0.09	0.05	0.02	0.01	0	0.02	0
SE	0.06	0	0.01	0.01	0.02	0.08	0.03	0.02	0.01	0	0.01	0
Range	00.01 – 0.04	0	0 – 0.04	0 – 0.04	0 – 0.12	0 – 0.4	0 – 0.16	0 – 0.08	0 – 0.04	0	0 – 0.04	0
	Natural infection (N = 10)*											
	Pooled	**1**	**2**	**3**	**4†**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Mean (lpg)	1.11	0.31	0.76	0.43	1.09	0.62	0.22	0.63	0.54	0.29	0.73	0.66
SE	0.41	0.13	0.33	0.14	0.53	0.14	0.05	0.23	0.14	0.06	0.23	0.33
Range	0.1 – 4.5	0 – 1.4	0 - 3.6	0.04 – 1.6	0 – 5.6	0 – 1.4	0.08 – 0.6	0 – 1.8	0 – 1.6	0 – 0.6	0 – 2.2	0 – 3.5

1. Tongue 2. Pterygoid 3. Sternomastoid 4. Tricep 5. Intercostal 6. Longissimus complex 7. External tibial flexor 8. Tail–Longissimus caudalis 9. Tail–Caudal femoral 10. Tail–Dorsal biopsy 11. Tail–Ventral biopsy. Lpg = larvae per gram of muscle

SE = Standard Error of Mean

*****Adapted from La Grange *et al*., 2013 excluding animal number CS 08.

† Depicts muscle with highest average larvae per gram.

Table 3.3. Mean (lpg muscle) distribution of *Trichinella zimbabwensis* larvae in grouped muscles of experimentally and naturally infected Nile crocodiles (*Crocodylus niloticus*).

High Infection

	Cranial[a]	**Abdominal[b]**	**Caudal[c]**
Mean (lpg)	10.14	36.26	14.82
SE	2.92	9.92	1.76
Range	0.5 – 18.9	7.4 - 65	9.4 – 18.6

Medium infection

	Cranial	**Abdominal**	**Caudal**
Mean (lpg)	2.03	5.62	1.29
SE	1.63	3.32	0.64
Range	0.04 – 8.5	0.36 – 16.6	0.24 – 3.5

Low infection

	Cranial	**Abdominal**	**Caudal**
Mean (lpg)	0.02	0.2	0.02
SE	0.01	0.12	0.02
Range	0 – 0.04	0 – 0.04	0 – 0.08

Natural infection*

	Cranial	**Abdominal**	**Caudal**
Mean (lpg)	1.51	2.56	2.22
SE	0.59	0.75	0.69
Range	0.08 – 6.6	0.04 – 8.2	0.3 – 7.9

Lpg = larvae per gram of muscle sample;

Cranial[a] muscles include tongue, pterygoid and sternomastoid;

Abdominal[b] muscles include tricep, longissimus complex, intercostal and external tibial flexor;

Caudal[c] muscles include longissimus caudalis, caudal femoral, dorsal biopsy and ventral biopsy

*Adapted from La Grange *et al*., 2013 excluding animal number CS 08.

Table 3.4. Distribution of *Trichinella zimbabwensis* larvae in tail musculature of Nile crocodiles (*Crocodylus niloticus*) sampled through necropsy and biopsy.

High Infection (N = 5)				
	Necropsy		**Biopsy**	
	Longissimus caudalis	Caudal femoral	Dorsal	Ventral
Mean (lpg)	3.02	5.34	4.32	2.14
SE	0.63	1.55	1.08	0.29
Range	1.7 – 5.3	1.6 – 9.4	2.5 – 8.4	1.5 – 3.2
Medium infection (N = 5)				
	Necropsy		**Biopsy**	
	Longissimus caudalis	Caudal femoral	Dorsal	Ventral
Mean (lpg)	0.27	0.14	0.44	0.45
SE	0.17	0.12	0.14	0.27
Range	0 – 0.9	0 – 0.6	0.2 – 0.9	0 – 1.1
Low infection (N = 5)				
	Necropsy		**Biopsy**	
	Longissimus caudalis	Caudal femoral	Dorsal	Ventral
Mean (lpg)	0.08	0	0.02	0
SE	0.08	0	0.01	0
Range	0 – 0.04	0	0 – 0.04	0
Natural infection (N = 10)*				
	Necropsy		**Biopsy**	
	Longissimus caudalis	Caudal femoral	Dorsal	Ventral
Mean (lpg)	0.54	0.29	0.73	0.66
SE	0.14	0.06	0.23	0.33
Range	0 – 1.6	0 – 0.6	0 – 2.2	0 – 3.5

Lpg = larvae per gram of muscle; SE = Standard Error of Mean; *****Adapted from La Grange *et al*., 2013 excluding animal number CS 08.

Chapter 4

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

4.1 Abstract

Several studies have reported on the influence of *Trichinella* spp. infection on biochemical parameters in experimental studies involving mammalian hosts. To date no controlled studies have been conducted to assess the influence of *T. zimbabwensis* infection on biochemical parameters in crocodiles. Fifteen crocodiles were randomly divided into three cohorts of five animals each to represent high, medium and low infection cohorts represented by 642, 414 and 134 larvae/kg bodyweight respectively. The biochemical parameters assessed were blood glucose, creatine phosphokinase (CPK), lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT). The humoral immune response to *T. zimbabwensis* infection was evaluated in all three cohorts by way of indirect ELISA. The results showed deviations from normal parameters of blood glucose, CPK, LDH, AST and ALT when compared to reported levels in uninfected reptiles. Contrary to studies involving mammals, hypoglycaemia was not observed in the infected cohorts in this study and peak values of blood glucose were observed on day 56, 49 and 42 p.i. in the high, medium and low infection cohorts respectively. Peak values of CPK were observed on day 35 p.i. in all three cohorts. Peak values of LDH and AST were observed at day 56, 49 and 42 p.i. in the high, medium and low infection cohorts respectively. Peak ALT values were reached on day 56 p.i. in the high infection cohort and on day 28 p.i. in both the medium and low infection cohorts. There were no correlations observed between the biochemical parameters and infection intensity. No significant differences in the titre levels between the three cohorts were observed. Peak antibody titres were reached on day 49 p.i. in the medium infection cohort and on day 42 p.i. in both the high and low infection cohorts. Infection intensity could not be correlated with the magnitude of the humoral response or time to seroconversion. 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.

4.2 Introduction

Trichinella larvae invade muscle tissue and results in 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 an increase of the cell membrane permeability and together these factors result in serum pervading into the adjacent tissue (Kocieska, 2000). This leads to an increase of creatine phosphokinase (CPK), lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT) in the blood (Kocieska, 2000).

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* spp. 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 p.i. (Nishina & Suzuki, 2002).

Serum levels of CPK, LDH and AST are used in diagnostic procedures to detect *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 *T. pseudospiralis*. Increased serum levels of ALT in pigs infected with *T. spiralis* have also been reported previously (Ribicich *et al*., 2007). However, elevated serum levels of these enzymes are not necessarily indicative of *Trichinella* infection as there are other causes (Wisniewska, 1970; Koudela & Schanzel, 1980; Tassi *et al*., 1995; Srivastava & Chosdol, 2007; Ribicich *et al*., 2007). Elevated CPK and LDH levels are also noted in cases of myocarditis and damage to heart musculature is possible as a result of trichinellosis (Tassi *et al*., 1995; Wisniewska, 1970). Similarly, elevated levels of ALT are known indicators of hepatic failure (Ribicich *et al*., 2007). Increases in LDH and CPK could also not be correlated with clinical severity of trichinosis in human patients and AST levels are not always increased (Kocieska, 2000). Comparisons between rats and human patients in one study found that increases in enzyme levels are dependent on the individual response from the host rather than being correlated with infection intensity or clinical severity (Wisniewska, 1970).

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

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

Studies in mammals have shown that *Trichinella* spp. infecting dose influence the period from infection to sero-conversion and that an initial infection with high numbers of larvae correlates with earlier sero-conversion (Gamble *et al*., 1988; Gottstein *et al*., 2009) and earlier larval establishment (Soulé *et al*., 1989). In a study involving rats infected with *T. spiralis* expulsion of adult worms started between eight to 10 days p.i. and in some cases continued until 28 days p.i. (Love *et al*., 1976). Once ingested, larvae mature in the host intestine within 36-48 hours and develop into adults within four to five days (Fabre *et al*., 2009). This short developmental period does not allow the host to launch an effective immune response against the adult worms until they have reproduced (Fabre *et al*., 2009) which explains the delay in sero-conversion and the subsequent effective establishment of newborn larvae. Gottstein *et al*. (2009) reported that anti-*Trichinella* IgG can be detected in animals between two and three weeks p.i. and that the disappearance of antibodies could be correlated with decreased numbers of muscle larvae in the host.

Information is scanty concerning the antibody response of the host against muscle stages of the parasite but a mixed isotype response of IgG1, 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 a diagnostic tool in animal trichinellosis has been evaluated. Enzyme immunoassay tests (Soule *et al*., 1989; Gamble *et al*., 1996) and indirect immunofluorescence assays have been conducted in goats and horses (Soule *et al*., 1989; Reina *et al*., 1996). Immunoassays were reported to be useful in horses but the study animals were all euthanized at 12 weeks post infection (Gamble *et al*., 1996) and 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). However, the practical application of these techniques is limited since specific

antibodies against *Trichinella* do not persist indefinitely and can only be detected for limited periods following infection. Dzik (2006) reviewed the different methods employed by helminth parasites to evade the immune response and reported several molecules released by *T. spiralis* as a strategy to evade the immune system of host. In the case of reptiles, other 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 (Brown *et al*., 2001; Ludovisi *et al*., 2013).

Since *T. 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, personal communication, 2008). An experimental study to determine the feasibility of use of ELISA for the detection of *T. zimbabwensis* infection in crocodiles has been conducted where results show that antibody titres decreased and eventually disappeared altogether (Ludovisi *et al*., 2013). In the study by Ludovisi *et al*. (2013), initial levels of infection were controlled in all the experimental study animals but the effect of infection intensity on the persistence of antibody titres could not be established in some animals as larvae failed to establish (La Grange, *unpublished*). The objective of this study was to determine the effect of *T. zimbabwensis* infection intensity on the levels of blood glucose, AST, ALT, CPK and LDH in experimentally infected crocodiles and the influence of infection intensity on the humoral immune response of the infected crocodiles.

4.3 Materials and Methods

To assess the influence of *Trichinella zimbabwensis* larvae on selected biochemical parameters, fifteen crocodiles were randomly divided into three cohorts of five animals each to represent high infection (642 larvae/kg of body weight), medium infection (414 larvae/kg of bodyweight) and low infection (134 larvae/kg of bodyweight) cohorts.

4.3.1 Source and preparation of infective material

Infective material was sourced from a crocodile experimentally infected with *T. zimbabwensis*. Muscle tissue was collected from various sites, minced and thoroughly mixed by hand (in protective clothing) using a ladle to stir the minced material to a homogenised sample. 100 Grams of the homogenised sample was processed by artificial digestion and infection level was determined to be 30 larvae per

gram (LPG) of the homogenized sample. Infective material for each individual animal was separately packaged and refrigerated at 4°C until the day prior to infection.

4.3.2 Infection of experimental animals

Infective material was removed from the refrigerator the evening before infection to allow it to reach room temperature.

In order to allow adequate time to conduct euthanasia of the experimental animals and testing of samples for each cohort of animals, the cohorts were infected at weekly intervals.

The animals were starved for at least 72 hours prior to infection to facilitate the infection process and prevent regurgitation of infective material caused by overfilled stomachs.

In order to administer the infective material, each animal was restrained by hand, the eyes and mouths taped shut and the animal placed on a table and secured by hand. The tape around the mouth was removed and a perspex tube 20mm long with a diameter of 40mm was placed in the tip of animal's mouth and taped into position to facilitate the insertion of the stomach tube and to ensure that the tube is not crushed. A thin dowel stick was used to open the gular valve at the back of the mouth and pressed flat against the mouth floor to allow insertion of a stomach tube. A stomach tube (700mm in length, 20mm in diameter) was inserted through the opening of the perspex tube into the oesophagus and carefully pushed into the opening of the stomach (Plate 2.1).

Once the tube was in the stomach, the animal was held in a vertical position and a short stem funnel was attached to the stomach tube and the required amount of infective material was trickled down into the tube and pushed towards the stomach with a dowel stick. Once all of the infective material was administered, a small amount of water was used to wash down any material that may have been caught on the sides of the tube. The animal was then placed on the ground and the tape and perspex tube removed. The animals were continuously monitored for at least thirty minutes following infection to ensure that they do not regurgitate.

4.3.3 Collection of serological samples

In order to evaluate the influence of infection on blood glucose and levels of CPK, LDH, ALT and AST, blood was collected from each of the animals on a weekly basis from the date of infection (Day 0) until eight weeks post infection (Day 56). Approximately eight

ml of blood was collected from each animal. A 10ml syringe and a 21 gauge needle were used to collect blood from the supra-vertebral sinus located caudally from the cranium. Blood was allowed to clot and the samples centrifuged at 10 000 rpm for 15 minutes to separate the serum.

Plate 4.1 Collection of blood from the supra-vertebral sinus in a Nile crocodile (*Crocodylus niloticus*).



4.3.3.1 Preservation of samples

For the detection of antibodies 2ml of 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.

4.3.4 Testing of blood glucose and serological samples

4.3.4.1 Glucose testing

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

4.3.4.2 Testing for antibody titres

Sera preserved in 0,01% merthiolate was referred to the International *Trichinella* Reference Centre in Rome, Italy for testing according to the following procedure;

Excretory/ secretory antigens (ESA) were produced by maintaining *T. zimbabwensis* larvae derived from infected mouse carcasses in supplemented RPMI 1640 medium for 18 hours in accordance with a validated protocol based on Gamble *et al*. (1988). (www.iss.it/binary/crlp/cont/ELISA%20Web%20SITE.1177083731.pdf). Anti-crocodile sera were also raised from six non-infected crocodiles. 50µg or 100 µg Serum was administered to four month old New Zealand rabbits with Freund's adjuvant. The

immune responses of the rabbits were evaluated by immunoelectrophoresis and those with the highest precipitation arcs used for the production of anti-crocodile IgG. The selected sera were treated with caprilic acid (Steinbusch and Audran, 1969) to produce the anti-crocodile IgG which was then conjugated with horseradish peroxidise (Nakane and Kawaoi, 1974). The conjugate specificity was determined by direct ELISA using serum from crocodiles, pigs, horses and rabbits to coat the microtitre plates. Only the sera derived from crocodiles reacted with the rabbit anti-crocodile IgG peroxidise. The ELISA was modified from a previously published protocol (Dawo and Mohan, 2007). The plates were coated with 5 µg/mL of ESA in 0.1 M carbonate buffer (pH 9.6) and incubated at 37°C for 60 minutes before rinsing three times for 5 minutes with 0.05% Tween 20 in phosphate buffered saline (PBS) with pH 7.2. PBS with 1 % BSA and 0.05% Tween 20 was used as blocking solution and the crocodile sera diluted to 1/50 in this solution. The highest difference in optical density (OD) values between samples collected at day 0 and the samples collected at weekly intervals were found at a dilution 1/80 which was also used as the optimal working dilution for the anti-crocodile IgG peroxidise. Tetramethylbenzidine was added as substrate and the OD values were read at 450nm. All the samples were tested in duplicate.

4.3.4.3 Testing of enzyme levels

Sera frozen at -18°C were submitted to the biomedical research laboratory at the University of KwaZulu-Natal, Westville campus, Durban, for testing. All of 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.

4.4 Data analysis

Weekly mean blood glucose and serum concentrations of CPK, LDH, ALT and AST were determined for each cohort 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 (Huchzermeyer, 2003, Lovely *et al*., 2007, Botha, 2010, 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 (Millan & Janmaat, 1997; Diethelm & Stein, 2005, Padilla *et al*., 2011). Reference ranges for several crocodilian species are shown in Table 4.1. Enzyme values from day seven p.i. were expressed as a

percentage of the normal values recorded on Day 0 and calculated as follows: Pv = (Iv / D0)*100 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 was 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 to determine relationships between biochemical parameters and infection intensity.

4.5 Results

4.5.1 Blood glucose

Overall mean concentration of blood glucose for all three experimental cohorts are shown in Table 4.2. In the high and medium infection cohorts initial peaks in blood glucose values were observed on day 14 and seven p.i. respectively followed by a slight decrease in the following week. However, thereafter, blood glucose concentrations in the high infection cohort increased to 130% on day 28 p.i. from the initial concentration on day 0 and to174% on day 56 p.i. Similarly concentrations increased in both the medium and low infection cohorts to 117% and 110% for day 28 and 149% and 138% for day 56 respectively. In the low infection cohort, concentrations initially decreased by 6% on day 7 p.i. The mean changes in blood glucose concentrations are shown in Figure 4.1. Analysis of variance revealed significant differences in blood glucose concentrations on day 28 p.i. between the high and low infection cohorts and between the medium and low infection cohorts with significantly higher concentrations in the high and medium infection cohorts compared to the low infection cohort. No significant differences within or between any of the experimental cohorts were observed at any other period within the experiment. No correlation between blood glucose and intensity of infection was observed.

4.5.2 Alanine transaminase (ALT)

Mean values of ALT for the three experimental cohorts are shown in Table 4.3. The highest increase of ALT was observed in the high infection cohort on day 28 and 35 p.i. and was 42% higher than the initial concentrations on day 0. Increases in ALT in the medium and low infection cohorts were less pronounced and reached peak values of 8% and 11% higher than day 0 at 42 and 28 days p.i. respectively. Analysis of variance showed no significant difference in the increases in ALT concentrations among the

cohorts. However, mean ALT concentrations were correlated with mean blood glucose concentrations in the high infection cohort ($R^2 = 0.9$, $P < 0.05$).

4.5.3 Aspartate transaminase (AST)

Mean values of AST for all three experimental cohorts are shown in Table 4.4. Analysis of variance showed mean AST values in the low infection cohort to be significantly higher on day 14 p.i. and day 42 p.i. ($P < 0.05$) compared to the high infection cohort.

Percentage increases in AST values for all three experimental cohorts are shown in Figure 4.2. Values decreased to approximately 95% from the initial level on day 0 to day seven p.i. but subsequently increased to 135% on day 35 p.i. for the high infection cohort. Maximum values were reached on day 56 p.i. AST values similarly decreased in both the medium and low infection cohorts to 89% and 98% for day seven p.i. respectively.

In the medium infection cohort AST values initially reached a peak on day 28 p.i. AST levels in this cohort decreased again until day 42 p.i. before increasing again and finally reached its maximum peak value on day 49 p.i. 247% higher than on day 0. In the low infection cohort however, only a single peak was observed on day 42 p.i. The percentage increase of AST in the low infection cohort on day 42 p.i. was significantly higher than that observed in the medium infection cohort ($P < 0.05$).

Despite significantly higher mean AST values in the low infection cohort on day 14 p.i. and day 42 p.i. ($P < 0.05$) compared to the high infection cohort, the percentage increase on these days was not significant. On day 56 p.i. however, AST values in the high infection cohort increased to significantly higher levels than those observed in both the medium and low infection cohorts ($P < 0.05$).

4.5.4 Creatine phosphokinase (CPK)

Overall mean values of CPK for all three experimental cohorts are shown in Table 4.5. Analysis of variance showed significant differences in changes of mean CPK values between the high and low infection cohorts from day 0 until day 14 p.i. of the trial with percentage increases in CPK values in the low infection cohort being significantly higher ($P < 0.05$). Mean CPK values were significantly increased in the low infection cohort compared to the medium infection cohort on day seven, 42, and 56 p.i. ($P < 0.05$). Similarly CPK in the low infection cohort increased to significantly higher levels than those recorded for the high infection cohort on days 42 and 56 p.i. ($P < 0.05$). The

mean increase in CPK value was also significantly higher in the medium infection cohort compared to the high infection and low infection cohorts on day 28 p.i. ($P < 0.05$). 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 cohort but a significant negative correlation was observed with overall infection intensity in this cohort ($R^2= -0.9$, $P < 0.05$). Mean CPK was negatively correlated with mean blood glucose in the medium infection cohort ($R^2= -0.9$, $P < 0.05$). No significant correlation could be established between infection dose or overall infection intensity and peak CPK values.

Percentage increases in CPK values for all three experimental cohorts are shown in Figure 4.3. CPK values decreased again from day 35 p.i. in all three cohorts and by day 56 p.i. reached levels slightly lower than those recorded on day 0 in the medium and low infection cohorts. CPK values reached its lowest point on day 49 p.i. in the high infection cohort and increased again to its second highest value on day 56 p.i. The low infection cohort showed significantly lower values of CPK from day 0 compared to the high and medium infection cohorts.

CPK values decreased in the medium infection cohort to approximately 66% from the initial concentration on day 0 to Day 28 p.i. In the high infection cohort mean CPK values showed an increase between day 0 and day 14 p.i. to approximately 125% of the initial concentration. This was followed by a decrease between day 14 and day 21 p.i. (32%). From day 21 p.i. CPK values increased by 479% higher than the value recorded on Day 0 in the high infection cohort. Similarly, CPK values increased in the medium infection cohort from day 28 p.i. and were 279% higher than the initial concentration. For the low infection cohort the increase in CPK values from day 28 p.i. reached a peak value 776% higher than normal at day 35 p.i.

4.5.5 Lactate dehydrogenase (LDH)

Overall mean values of LDH for all three experimental cohorts are shown in Table 4.6. LDH values in all three experimental cohorts gradually increased until day 35 p.i. This gradual increase was marked by a sharp increase to reach peak values on day 42 p.i., 49 p.i. and 56 p.i. for the low, medium and high infection cohorts respectively. These trends were similar to the trends observed for AST values in all three cohorts.

Mean increase in LDH was significantly higher in the low infection cohort on day 28 and 35 p.i. compared to the medium infection cohort ($P < 0.05$). Mean increases in LDH values were significantly higher in the low infection cohort compared to the high

infection cohort on day 35 p.i. ($P < 0.05$). Mean LDH concentrations in the low infection cohort reached significantly higher values on day 21 and 42 p.i. compared to the high infection cohort ($P < 0.05$). Mean increase in values of LDH was significantly higher in the high infection cohort on day 56 p.i. compared to the medium and low infection cohort ($P < 0.05$). LDH values were also significantly higher in the medium infection cohort compared to the high infection cohort on day 28 and 49 p.i. ($P < 0.05$). Mean LDH values were also significantly higher in the medium infection cohort on day 56 p.i. compared to the low infection cohort ($P < 0.05$).

Mean LDH values were negatively correlated with blood glucose in the medium infection cohort (R^2= -0.9, $P < 0.05$). A significant correlation was also observed between mean LDH concentrations and infection dose in the low infection cohort (R^2= 0.9, $P < 0.05$).

Percentage increases in LDH values for all three experimental cohorts are shown in Figure 4.4. For the high infection cohort LDH values increased to 553% from normal concentrations on day 0. Peak values representing a 447% and 389% increases from normal concentrations were observed in the medium and low infection cohorts. No direct correlation could be established between peak LDH values and intensity or infection dose.

4.5.6 Indirect ELISA

Seroconversion was observed in all of the experimental animals. Mean changes in anti-*Trichinella* IgG for all three the experimental cohorts are shown in Figure 4.5. Analysis of variance showed no significant differences in the titre levels between the three cohorts despite the fact that mean optical density (OD) values in the high infection cohort were lower compared to the medium infection cohort. Mean OD values were also lower in the medium infection cohort compared to the low infection cohort. No significant correlation between infection intensity and magnitude of the humoral response could be established. Peak antibody titres were reached at day 42 p.i. in both the high and low infection cohorts whereas the medium infection cohort reached its highest value on day 49 p.i.

Analysis of variance however showed significant differences between the cohorts on day seven, 21 and 42 p.i. On day seven p.i. OD values were significantly lower in the high infection cohort ($P < 0.05$) compared to the medium and low infection cohorts. On day 21 p.i. OD values in both the high and low infection cohorts were significantly lower than in the medium infection cohort ($P < 0.05$). On day 42 p.i. OD values in the low

infection cohort were significantly higher compared to the high infection cohort. Kinetics of the antibody response (Figure 4.5) show mean seroconversion in the high infection cohort to have been delayed by a week compared to the medium and by two weeks compared to the low infection cohort. However, no significant correlation could be established between infection intensity and time to seroconversion.

4.6 Discussion

4.6.1 Influence of infection intensity on Blood glucose

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

Wu *et al*. (2009) reported an initial drop in blood glucose levels between day eight and 28 p.i. 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 13 and 18 days p.i for *T. spiralis* and *T. pseudospiralis* respectively and that *T. pseudospiralis*-infected mice took longer to recover to normal. Similarly, mice infected with *T. spiralis* in other studies also showed hypoglycaemia at 10 days p.i. (Nishina & Suzuki, 2002; Nishina *et al*., 2004).

The initial decrease in blood glucose observed on day seven p.i. in the low infection cohort is consistent with results obtained from mice experimentally infected with *T. spiralis* (Nishina & Suzuki, 2002). In contrast, blood glucose levels increased between day 0 and seven p.i. in the high infection cohort and between day 0 and 14 p.i. in the medium infection cohort but subsequently decreased on day 14 p.i. in the medium infection cohort and on day 21 p.i. in the high infection cohort before increasing again. This suggests that the initial level of infection plays an important role in determining the time from infection until blood glucose levels are decreased.

Modulation of the insulin pathway by the parasite is considered the cause of hypoglycaemia and blood glucose levels corresponds to larval growth (Wu *et al*., 2009). This hypothesis is supported by results from this study. 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 and 18 p.i. respectively and that in the case of the latter, recovery time from hypoglycaemia was longer. These differences suggest parasite specific mechanisms to be responsible. However, the initial rise in blood glucose levels observed in the high and medium infection cohorts 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 p.i., rarely reaching 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 one might hypothesize that crocodiles possess physiological adaptations that allow them to up-regulate blood glucose concentrations in accordance with the existing demand. Dolphins are able to switch between a normal and hyperglycaemic state between 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 the result of stress associated with capture and restraint (Smith & Marais, 2004). However, the increase in blood glucose levels observed in the medium and high infection cohorts between the fourth and sixth week p.i. and the third and fifth week p.i. in the low infection cohort appear to coincide with the arrival and infiltration of newborn larvae (NBL) in the muscle tissue. In this study minimum and maximum blood glucose levels were not reached at the same time in the three cohorts but were marked by a one week delay between each cohort with the low infection cohort reaching 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 in the host circulation. Large larval burdens in the blood circulation could

potentially restrict blood flow in vital organs or acute anaphylaxis causing host fatality which ultimately is detrimental to survival of the parasite.

4.6.2 Influence of infection intensity on alanine transaminase (ALT)

Mean ALT of the experimental crocodiles on Day 0 compared favourably with normal reference ranges reported for Nile crocodiles by other authors (Lovely *et al*., 2007; Botha, 2010). 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, 2005]. Increased ALT activity is normally more indicative of liver disease and according to Srivastava & Chosdol (2007), increase in the activity of this enzyme is rarely noted in other cases.

Peak values of ALT were not reached simultaneously in the three cohorts. 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 result 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 cohorts and 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.

4.6.3 Influence of infection intensity on aspartate transaminase (AST)

Mean AST of the experimental crocodiles on Day 0 compared favourably with normal reference ranges reported for Nile crocodiles by other authors (Lovely *et al*., 2007; Botha, 2010). The initial peaks in AST values observed in the medium and high infection cohorts on day 28 and day 35 p.i. respectively followed by their respective maximum values on day 49 and 56 p.i suggest that larvae were not released simultaneously from the small intestine. A similar pattern was also observed for CPK in the high infection cohort. In the medium infection cohort however the initial peak in AST on day 28 p.i. did not coincide with the peak observed in CPK.

In the low infection cohort only a single peak in AST was observed on day 42 p.i. suggesting that all the larvae reached the muscles simultaneously. The maximum values in AST coincided with the response observed in LDH. In contrast with ALT, the response of individual crocodiles in the different cohorts was also more uniform and

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 concentrations are higher than ALT and the ratio of AST:ALT is reported to be >1 (Srivastava & Chosdol, 2007). Normal values of these enzymes as recorded for the experimental crocodiles in this study support this and AST values were on average higher [31.92 international units per litre (IU/L)] than that of ALT (28. 67 IU/L). Srivastava & 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 (38.77 IU/L) exceeded those of AST (31.80 IU/L) over the course of the experiment.

4.6.4 Influence of infection intensity on creatine phosphokinase (CPK)

No reference ranges for CPK for Nile crocodiles have been reported to date. However, mean CPK of our experimental crocodiles on Day 0 fell well within normal reference ranges reported for American alligators (*A. mississippiensis*) and Dwarf caimans (*Paleosuchus palpebrosus*) as reported by (Diethelm & Stein, 2005).

The large, single peak increase in CPK concentrations in the low infection cohort on day 35 p.i. may correspond with the arrival of larvae and subsequent invasion of muscle tissue. It further suggests that the newborn larvae reached their respective sites simultaneously which resulted in a singular, large scale myopathy event. Analyses of the individual results confirmed that all five animals in this cohort reached peak CPK concentrations on day 35 p.i.

In the medium and high infection cohorts the increase in CPK concentration was not only less pronounced, but the subsequent decrease was more protracted compared to the low infection cohort and CPK values remained higher than those recorded on day 0 in both these cohorts.

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 though, peak concentrations corresponded with the arrival of new-born larvae in the muscles around the fifth week p.i.

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 actual fact was less prominent when compared to LDH and AST in this study.

According to Srivastava & Chosdol (2007) CPK levels may rise within three to six hours in humans following a heart attack and will return to normal levels within 12-48 hours if no further damage occurs. The results from this study appear to support this as was the case in the low infection cohort where CPK levels decreased immediately after its initial peak on day 35 p.i. The fact that CPK levels in the low infection cohort did not increase from their lowest levels on day 49 and 56 p.i. or persisted at concentrations higher than those observed on day 0, suggests that all of the larvae had invaded the host musculature. No new events of myopathy occurred after day 35 p.i. and thus CPK concentrations decreased to normal levels.

However, in the other two cohorts CPK did not reach normal levels. This would suggest that muscle damage continued beyond the peaks observed on day 35 p.i. and further supports the hypothesis that larvae may not have been released simultaneously in the medium and high infection cohorts. This further supports the hypothesis that development of some larvae may have been delayed. In the medium infection cohort the initial damage was more severe than was the case in the low infection cohort and thus the decrease in CPK levels were more protracted. In the high infection cohort however, not only was the initial damage caused by the larvae more severe, but if new larvae continued to be released from the intestine after day 35 p.i., this would explain the subsequent increase in CPK from day 49 p.i. which may have coincided with the damage caused by newly released larvae infiltrating muscle cells unaffected by the first wave.

The negative correlation observed between mean blood glucose and mean CPK in the medium infection cohort may be explained by the fact that CPK levels continued to decrease beyond day 49 p.i. following its initial peak on day 35 p.i. whereas blood glucose levels increased again after day 49 p.i. If a second wave of larvae was released in this cohort, as might be suggested by the rise in glucose levels on day 49 p.i., their numbers may have been too few to cause a significant increase in the already elevated CPK levels recorded on day 49 p.i.

In the high infection cohort however, the second wave of larvae resulted in a secondary increase in CPK levels that coincided with the increase in blood glucose which resulted in the positive correlation observed between blood glucose and CPK concentrations in this cohort.

4.6.5 Influence of infection intensity on lactate dehydrogenase (LDH)

No reference ranges for LDH could be found for Nile crocodiles and mean LDH of the experimental crocodiles on Day 0 fell well within normal reference ranges reported for Dwarf caimans (*Paleosuchus palpebrosus*) as reported by Diethelm & Stein (2005).

In horses infected with *T. spiralis*, increases in CPK, LDH and aldolase all peaked at the fifth week p.i. and LDH was found to be more sensitive than CPK and aldolase (Soule *et al*., 1989). The sensitive nature of LDH was also supported by results from this 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 appear not to coincide with muscle damage caused by the larvae, however serum concentrations of enzymes may be 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 activity of LDH and infection intensity was not always apparent when comparing individual animals of the same group. Variability between individual animals within the three experimental cohorts of this study could thus explain the statistical insignificance of the correlation analyses.

In guinea pigs infected with *Trichinella* spp. larvae, LDH continued to increase until six weeks p.i. (Koudela & Schanzel, 1980). This is supported by the results observed in the low infection cohort. The continued increase in LDH observed in the medium and high infection cohorts once again suggests that larval development and subsequently larval migration and establishment, may have been delayed in these cohorts.

Similar to CPK, LDH was also negatively correlated with blood glucose in the medium infection cohort. Although LDH concentrations increased beyond day 35 p.i., it was also marked by a sharp decrease immediately following its peak on day 49 p.i. The differences in time to reach peak LDH and AST values observed between the experimental cohorts 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).

4.6.6 Influence of infection intensity on humoral immune response

An indirect ELISA has been previously 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 p.i. 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 (Pozio *et al*., 2002; Vu Thi *et al*., 2010, Ludovisi *et al*., 2013). In crocodiles, interpretation of antibody titres is also complicated since 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 but indicated that the time period between infection and sero-conversion of the host was problematic in surveillance. The results from this study confirm this since the time from infection to sero-conversion ranged from 21 days p.i. to 56 days p.i.

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 if they had 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 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 (Pozio *et al*., 2002; Vu Thi *et al*., 2010; Ludovisi *et al*., 2013).

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.

In six of the crocodiles, antibody levels had decreased below positive values by day 56 p.i. The results revealed similar problems to those reported in mammals infected with

other *Trichinella* species and showed that in most cases, antibody titres could not be detected indefinitely (Soule *et al*., 1989; Gottstein *et al*., 2009; Ludovisi *et al*., 2013).

4.7 Conclusion

Hypoglycaemia as reported in studies in mice involving *T. spiralis* and *T. pseudospiralis* (Nishina & Suzuki, 2002; Wu *et al*., 2009) was not observed in crocodiles experimentally infected with *T. zimbabwensis* in this study. On average, blood glucose levels increased in all three experimental cohorts of this study and did not reach normal concentrations at any stage of the experiment. These results suggest that Nile crocodiles up-regulate blood glucose as the demand for energy is increased by the parasites.

This phenomenon requires further investigation 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 parameters of the host.

High variability in ALT changes between individual crocodiles support the finding that increased ALT activity is not reliable as an indicator for muscle damage and that increases in ALT is rarely observed in cases where damage to the liver is not involved (Srivastava & Chosdol, 2007). With exception of the high infection cohort, differences in ALT concentrations observed in the crocodiles can largely be attributed to variations in individual physiology of the crocodiles.

Observed responses in CPK and AST in the medium and high infection cohorts suggest that larvae may have been released in waves from the small intestine in these cohorts but not in the case of the low infection cohort.

CPK was more reliable as an indicator of muscle damage as its peak values correspond with the arrival of larvae in the muscle although this was not in agreement with reports by Wisniewska (1970) that attributed changes in CPK to individual responses rather than infection.

There was lack of correlation between infection intensity and changes in biochemical parameters and this is probably because other host factors not measured in the study such as hormone levels and the age of the animal as well as environmental factors

including temperature and season, could have impacted on the immune response of reptiles (Brown *et al*., 2001; Ludovisi *et al*., 2013).

The lack of information on normal reference ranges for haematological and biochemical parameters in Nile crocodiles should additionally be addressed in future research.

4.8 References

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Figure 4.1 Mean glucose increase in Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*.

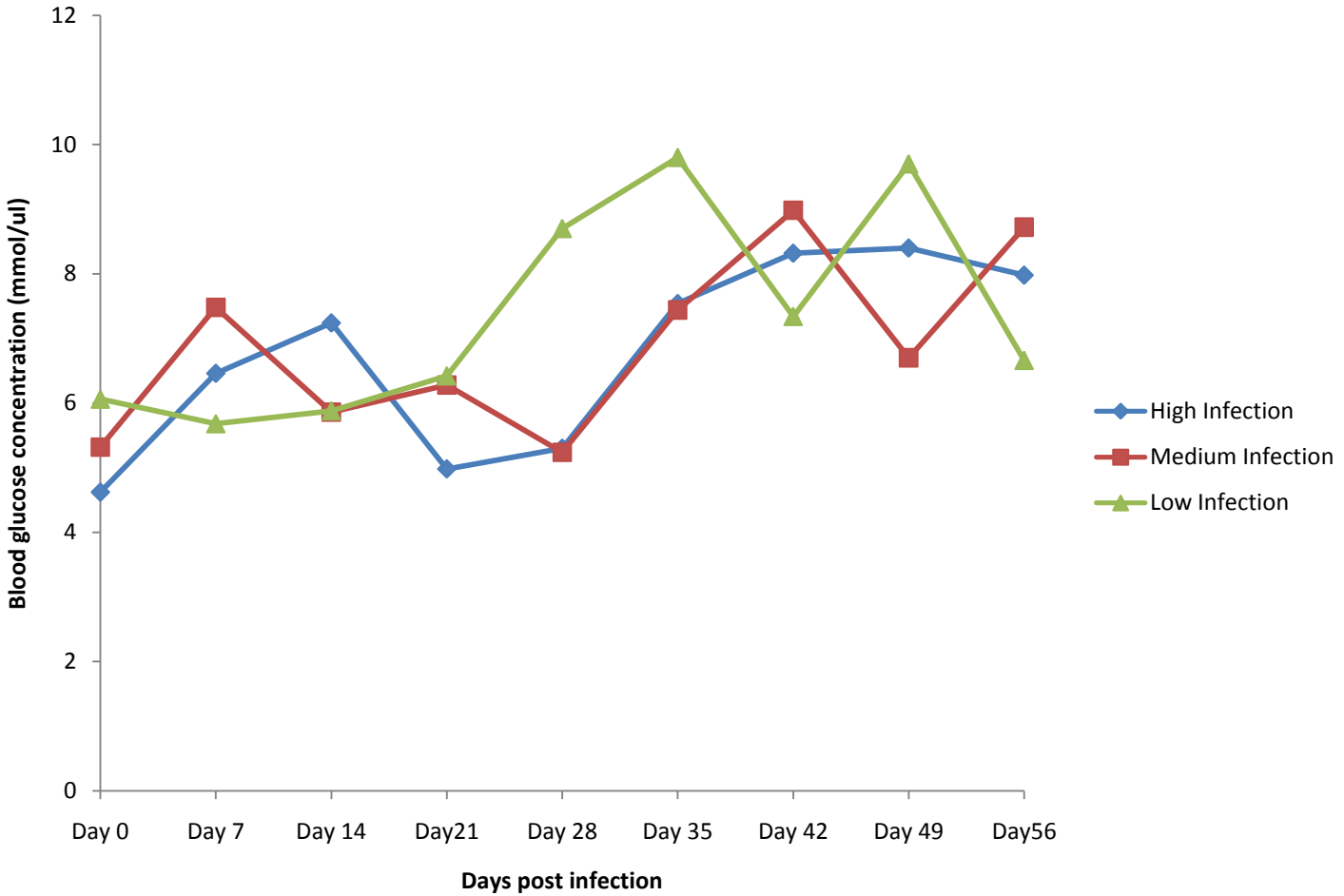
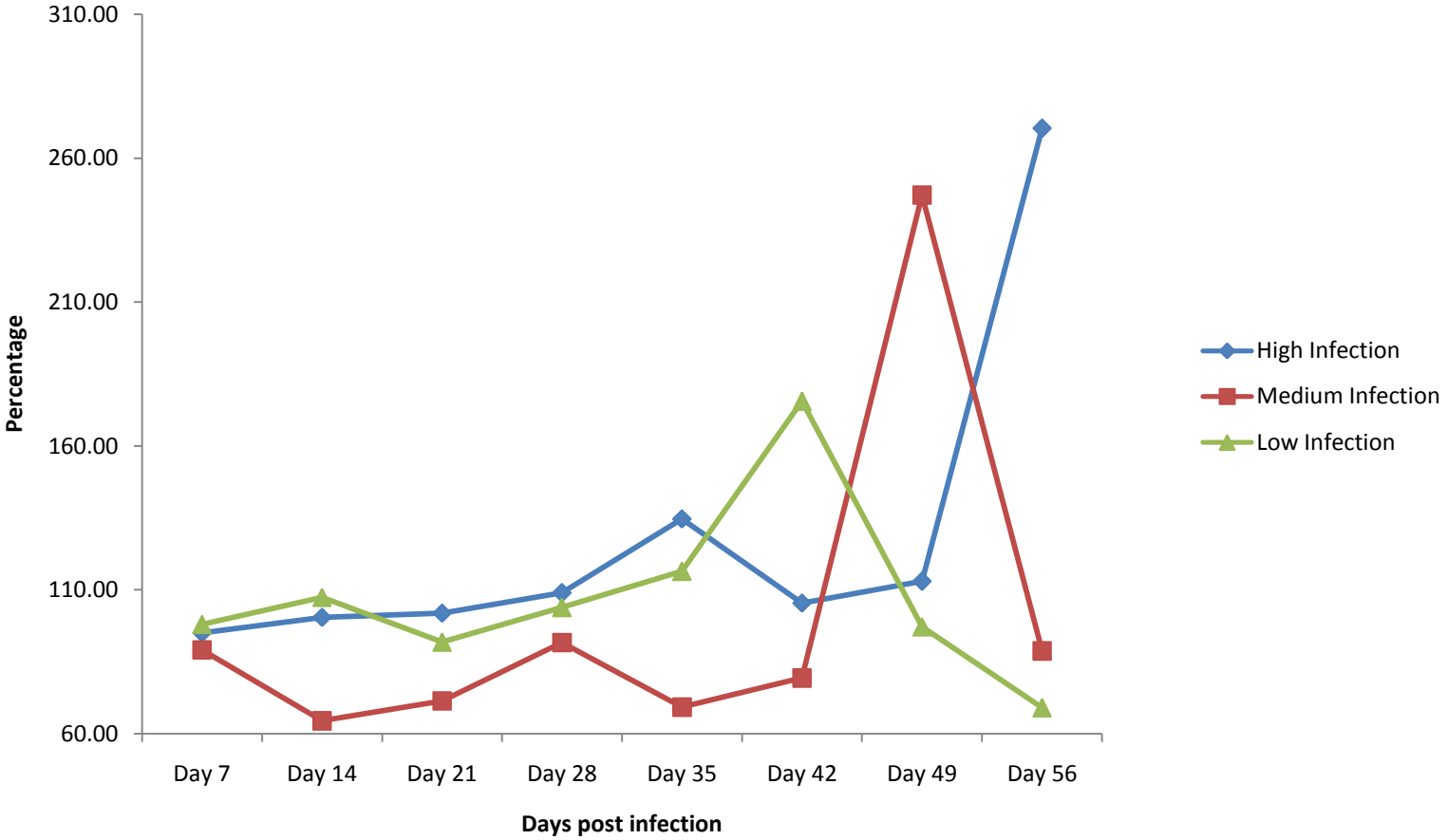
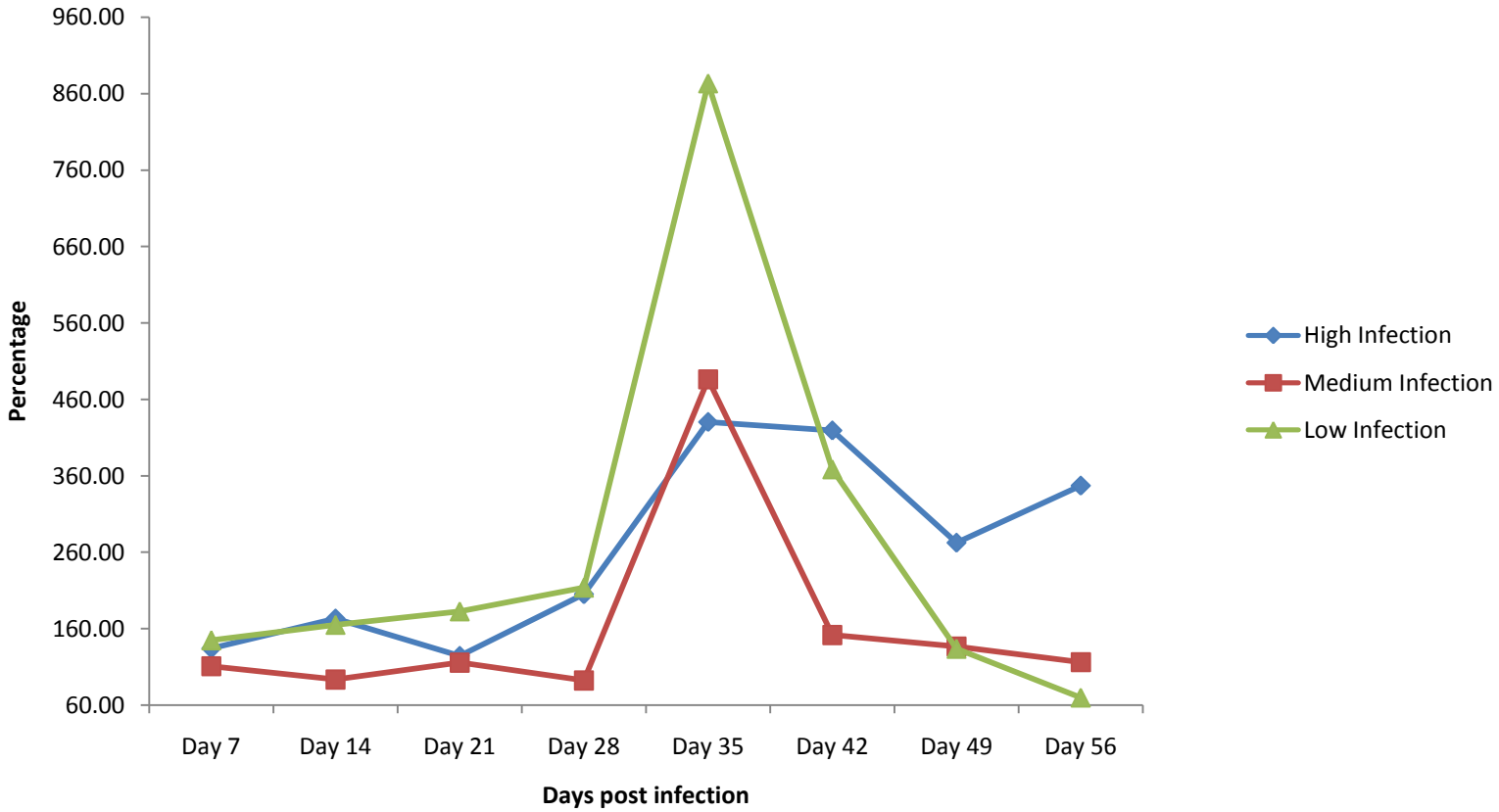


Figure 4.2 Percentage change in aspartate transaminase (AST) concentration in Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*.*



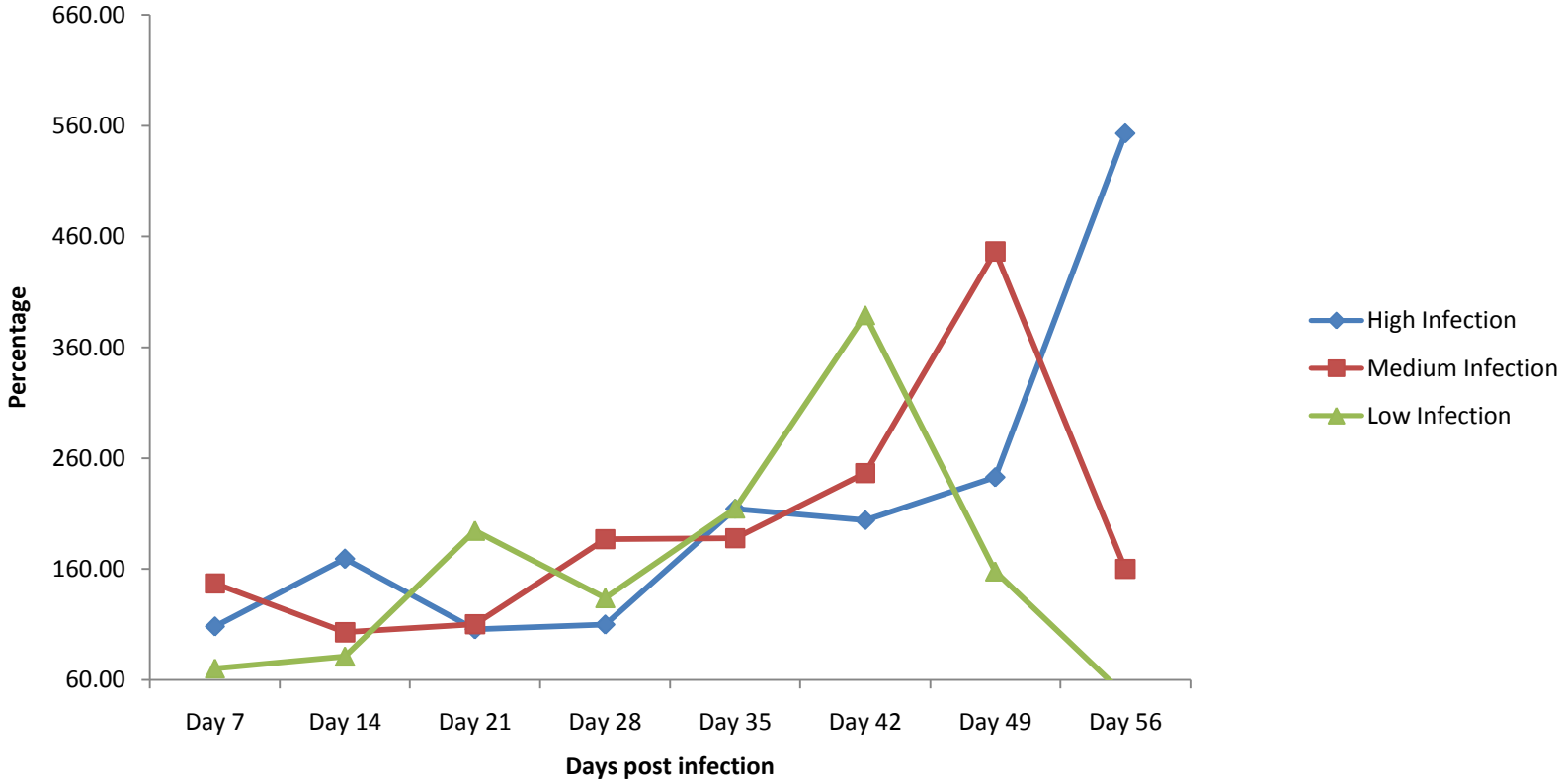
*Values are shown as a percentage of normal values recorded on Day 0.

Figure 4.3 Percentage change in creatine phosphokinase (CPK) concentration in Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis*.*



*Values are shown as a percentage of normal values recorded on Day 0.

Figure 4.4 Percentage change in lactate dehydrogenase (LDH) concentration in Nile crocodiles (*Crocodylus niloticus*) experimentally infected with T*richinella zimbabwensis*.*



*Values are shown as a percentage of normal values recorded on Day 0.

Figure 4.5 Mean change in anti-*Trichinella* IgG in Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis.*

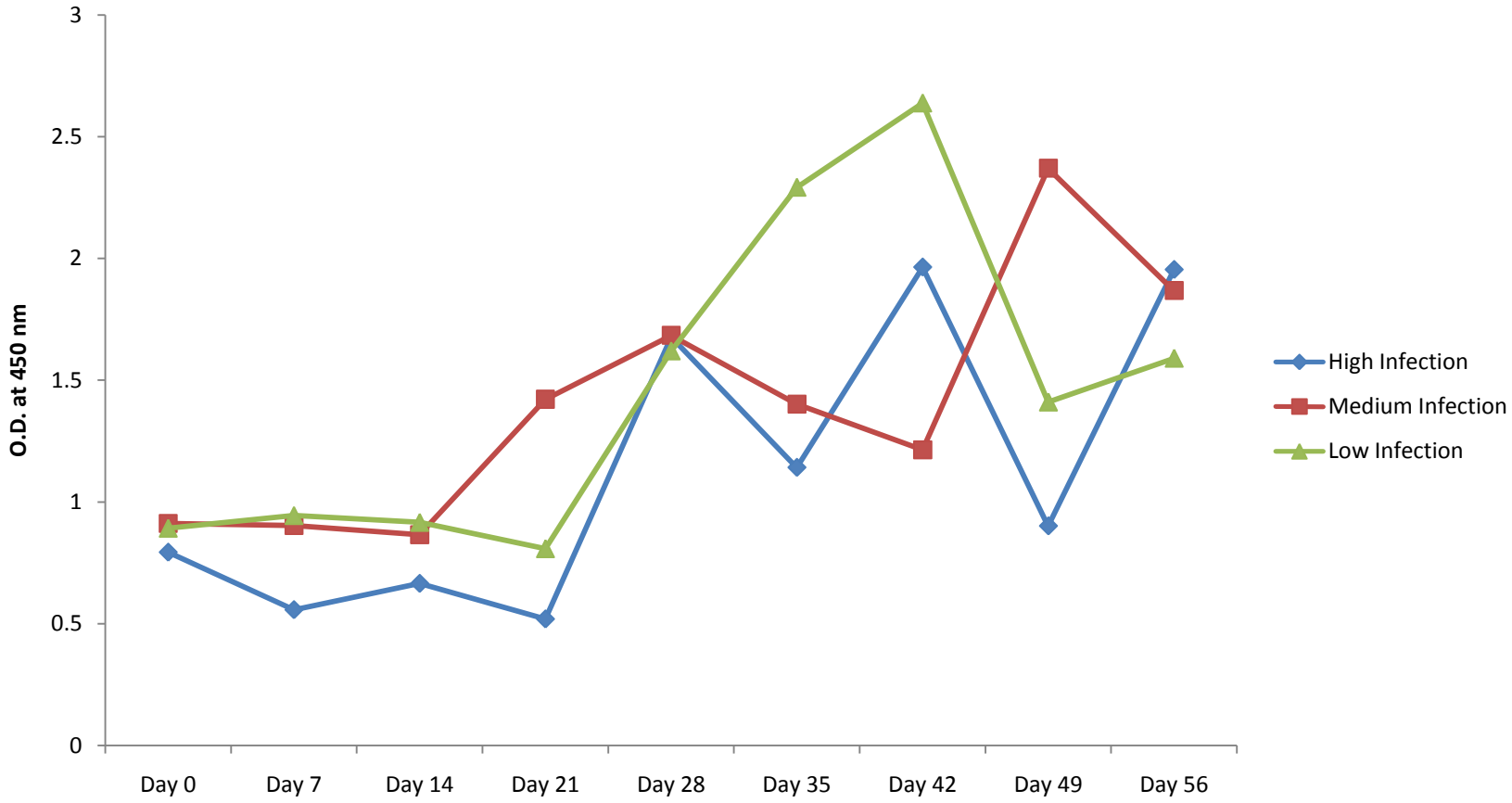


Table 4.1. Haematological and biochemical reference ranges in a variety of crocodilian species.

	Glucose	ALT	AST	CPK	LDH	Reference
Crocodylus niloticus	3.8 – 7.5 mmol/l	8-59	16-49	61.4 - 1893.5	638 - 3173	Current study
	81.6 mg/dl[a,b]					Padilla *et al*., 2011; Huchzermeyer, 2003
	3.3 – 4.8 mmol/l	15 - 69	14 - 211			Lovely *et al*., 2007
	3.87 – 5.68 mmol/l	13 - 30	24 -47			Botha, 2010
Alligator mississippiensis	92 mg/dl	46.05[a,b]	223.5[b]			Padilla *et al*., 2011[a]; Huchzermeyer, 2003[b]
	0 – 198 mg/dl	0 -154	0 - 700	0 - 8620	0 - 2000	Diethelm & Stein, 2005
	74mg/dl					Stacey & Whittaker, 2000
Paleosuchus palpebrosus	29 – 187 mg/dl	24 - 93	42 - 221	37 - 9890	80 - 6615	Diethelm & Stein, 2005
Crocodylus moreletti	64.25 – 74.85 mg/dl	16.68 -23.72				Padilla *et al*., 2011
	52.95 mg/dl					Huchzermeyer, 2003
Crocodylus porosus	4.5 – 12.1 mmol/dl[a,b,c]	11-51[a,b]	23 -157[a]			Millan & Janmaat, 1997[a]; Padilla *et al*., 2011[b]
	4.8 – 11.99 mmol/L	12 - 50	23 - 148			Richardson *et al*., 2002
	81 – 218mg/dl	11 - 51	23 - 157			Stacey & Whittaker, 2000
Caiman latirostris	81.82 mg/dl					Huchzermeyer, 2003[c]
Crocodylus palustris	55 -110 mg/dl					Huchzermeyer, 2003
	48 – 110mg/dl	28 - 97	23 - 70			Stacey & Whittaker, 2000
Tomistoma schlegelii	75.3 mg/ dl				426.2	Huchzermeyer, 2003
Crocodylus mindorensis	60 -168mg/dl					Stacey & Whittaker, 2000
Crocodylus acutus	101mg/dl					Stacey & Whittaker, 2000

Table 4.2. Mean blood glucose levels* in sera of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis.*

High infection (N = 5)(643 larvae/kg bodyweight)

	Overall Glucose	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Mean ± SE	6.76 ± 0.75	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	1.8 – 13.6	3.8 - 5.5	3.7 - 9.7	5.2 - 11.3	4.2 - 6.3	3.4 - 7.3	4.7 - 14.7	6.8 - 12.6	4.9 - 11.1	5.4 - 10.3

Medium infection (N = 5)(414 larvae/kg bodyweight)

	Overall Glucose	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Mean ± SE	6.89 ± 0.30	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	6.7 ± 0.31	8.72 ± 0.76
Range	6.29 – 8.06	4.7 – 6.8	5.5 – 9.4	4.6 – 7.8	5.4 – 7.4	3.8 – 6.8	6.9 – 8.4	8.0 - 10.3	5.5 – 7.3	6.2 - 10.4

Low infection (N = 5)(134 larvae/kg bodyweight)

	Overall Glucose	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Mean ± SE	7.36 ± 0.44	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	5.82 – 8.31	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

*Values are shown as millimol per microliter (mmol/ul)

Table 4.3 Mean alanine transaminase (ALT) levels* of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis.*

High infection (N = 5)(642 larvae/kg bodyweight)

	Overall ALT	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Mean ± SE	33.36 ± 8.64	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	22.0 – 29.63	8.0 – 16.0	14.0 – 32.0	5.2 - 11.3	4.2 - 6.3	3.4 - 7.3	4.7 - 14.7	6.8 - 12.6	4.9 - 11.1	5.4 - 10.3

Medium infection (N = 5)(414 larvae/kg bodyweight)

	Overall ALT	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Mean ± SE	41.49 ± 2.72	35.5 ± 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	7.56 – 48.56	32.0 – 40.0	25.0 – 46.0	25.0 – 50.0	28.0 – 54.0	39.0 – 65.0	27.0 – 51.0	36.0 – 61.0	28.0 – 48.0	25.0 – 58.0

Low infection (N = 5)(134 larvae/kg bodyweight)

	Overall ALT	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Mean ± SE	41.47 ± 1.43	33.4 ± 6.82	37.4 ± 7.92	37.6 ± 4.79	44.2 ± 7.15	47.2 ± 4.42	43.6 ± 7.65	44.2 ± 6.02	46.8 ± 10.33	38.8 ± 8.75
Range	38.56 – 43.78	23.0 – 59.0	28.0 – 69.0	22.0 – 48.0	23.0 – 68.0	32.0 – 57.0	14.0 – 57.0	25.0 – 55.0	8.0 – 69.0	12.0 – 59.0

*Values are shown as international units per litre (IU/L)

Table 4.4 Mean aspartate transaminase (AST) levels* of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis.*

High infection (N = 5)(642 larvae/kg bodyweight)

	Overall AST	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Mean ± SE	24.92 ± 1.59	21.75 ± 4.13	18.8 ± 1.28	20.0 ± 0.82	19.0 ± 2.53	20.8 ± 1.11	26.8 ± 5.44	18.75 ± 5.76	21.0 ± 1.92	53.2 ± 3.5
Range	25.11 – 30.14	16.0 – 34.0	15.0 – 22.0	18.0 - 22.0	14.0 - 26.0	17.0 – 24.0	9.0 - 42.0	6.0 - 32.0	14.0 - 25.0	44.0 – 65.0

Medium infection (N = 5)(414 larvae/kg bodyweight)

	Overall AST	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Mean ± SE	34.65 ± 2.18	38.5 ± 4.7	29.75 ± 7.55	25.0 ± 2.59	26.4 ± 1.03	29.5 ± 3.86	28.2 ± 4.04	28.2 ± 2.94	74.0 ± 16.71	31.4 ± 2.58
Range	35.22 – 41.0	27.0 – 48.0	15.0 – 48.0	19.0 – 33.0	24.0 – 30.0	21.0 – 37.0	18.0 – 37.0	19.0 – 34.0	20.0 – 120.0	26.0 – 39.0

Low infection (N = 5)(134 larvae/kg bodyweight)

	Overall AST	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Mean ± SE	35.82 ± 2.96	34.8 ± 4.07	31.4 ± 4.88	35.8 ± 3.34	30.6 ± 3.49	34.8 ± 3.87	39.4 ± 4.83	57.8 ± 8.09	33.6 ± 7.59	24.2 ± 4.09
Range	30.0 – 46.22	26.0 – 49.0	13.0 – 41.0	28.0 – 47.0	23.0 – 68.0	22.0 – 40.0	27.0 – 55.0	38.0 – 80.0	6.0 – 52.0	12.0 – 33.0

*Values are shown as international units per litre (IU/L)

Table 4.5 Mean creatine phosphokinase (CPK) levels* of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis.*

High infection (N = 5)(642 larvae/kg bodyweight)

	Overall CPK	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Mean ± SE	1497 ± 217	698 ± 145	770 ± 51	872 ± 268	599 ± 185	1507 ± 770	3343 ± 1205	2252 ± 522	1547 ± 874	1884 ± 673
Range	1132 – 2312	405 – 1099	577 – 854	454 – 1898	115 - 1084	269 – 4534	695 - 5064	1256 - 3652	414 - 5026	524 – 4410

Medium infection (N = 5)(414 larvae/kg bodyweight)

	Overall CPK	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Mean ± SE	1406 ± 113	1209 ± 230	1042 ± 129	802 ± 115	896 ± 201	802 ± 75	3377 ± 1029	1843 ± 539	1681 ± 701	998 ± 187
Range	1023 – 1678	448 – 1294	691 – 1384	541 – 1225	391 – 1449	592 – 1001	1701 – 6941	633 – 3684	690 – 4448	567 – 1684

Low infection (N = 5)(134 larvae/kg bodyweight)

	Overall CPK	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Mean ± SE	213 ± 23	93 ± 12	136 ± 41	148 ± 37	162 ± 34	187 ± 29	722 ± 128	297 ± 57	112 ± 20	63 ± 8
Range	130 – 272	61 – 123	54 – 254	75 – 275	86 – 271	148 – 302	266 – 1016	194 – 495	55 – 166	41 – 86

*Values are shown as international units per litre (IU/L)

Table 4.6 Mean lactate dehydrogenase (LDH) levels* of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis.*

High infection (N = 5)(642 larvae/kg bodyweight)

	Overall LDH	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Mean ± SE	1551 ± 137	827 ± 137	876 ± 184	1256 ± 271	764 ± 92	841 ± 55	1637 ± 318	1521 ± 367	1960 ± 375	4281 ± 240
Range	1408 – 2021	563 – 1348	288 – 1393	641 – 2180	455 - 991	752 – 1049	1181 - 2901	991 - 2959	1045 - 3021	3811 – 5194

Medium infection (N = 5)(414 larvae/kg bodyweight)

	Overall LDH	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Mean ± SE	2155 ± 277	1140 ± 320	1742 ± 321	1101 ± 157	1246 ± 324	2642 ± 704	1849 ± 392	2532 ± 469	5743 ± 1466	1395 ± 290
Range	1545 – 2966	724 – 2413	1011 – 2855	489 – 1360	409 – 2357	1033 – 4940	1156 – 3216	1223 – 3674	1004 – 9726	868 – 2527

Low infection (N = 5)(134 larvae/kg bodyweight)

	Overall LDH	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Mean ± SE	1854 ± 191	1592 ± 476	905 ± 158	944 ± 86	2179 ± 481	1729 ± 377	2416 ± 533	4853 ± 750	1532 ± 299	533 ± 191
Range	1512 – 2541	638 – 3173	344 – 1243	760 – 1179	1069 – 3737	915 – 2726	544 – 3793	3335 – 7403	704 – 2313	275 – 765

*Values are shown as international units per litre (IU/L)

Table 4.7 Mean change in anti- *Trichinella* IgG* in sera of Nile crocodiles (*Crocodylus niloticus*) experimentally infected with *Trichinella zimbabwensis.**

High infection (N = 5)(642 larvae/kg bodyweight)

	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
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 (N = 5)(414 larvae/kg bodyweight)

	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
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 (N = 5)(134 larvae/kg bodyweight)

	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
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 (O.D.) values at 450 nm.

† Values > 1.4 are considered positive.

Chapter 5

General Discussion and Conclusion

5.1 General Discussion

The zoonotic potential of *T. zimbabwensis* as supported by the clinical symptoms observed in experimentally infected, non-human primates (Mukaratirwa *et al*., 2001) necessitates research aimed at elucidating the distribution and epidemiology of this parasite.

However, human infections attributed to this parasite have never been documented and even infections attributed to other *Trichinella* spp. have been extremely rare comprising only 0.04% of all infections reported worldwide between 1986-2009 (Murrell and Pozio, 2011; Mukaratirwa *et al*., 2012). The correlation between rare disease incidence and customary practises in food preparation and religious laws that forbid pork consumption (Pozio *et al*., 2005) may apply to a proportion of the African population.

The lack of infrastructure in many countries, poor socio-economic development, rural areas closely related to vast wildlife reserves and the abundance of potential sylvatic hosts must caution one not to accept the current incidence or perceived risk of infection to be a realistic and true reflection of the actual situation. The concerns raised by Bengis and Veary (1997) in respect to the potential impact of certain cultural practises should be seriously considered.

The limited epidemiological data in respect of *T. zimbabwensis*, lack of official veterinary control for crocodile meat produced for local consumption and the disparate views on effective testing and control for the export markets leads to belief that the current knowledge and/or perception of the actual risk to humans in Africa represents merely a tip of an iceberg.

Results from this study show that *T. zimbabwensis* follow different predilection patterns in Nile crocodiles compared to those reported in caimans and varans (Pozio *et al*., 2004) but confirm the importance of leg musculature as predilection sites in sylvatic carnivores (Kapel *et al*., 1994, 1995). Altered predilection patterns were also observed between high and low infection intensities from this study and similar alterations were reported by Serrano & Pérez-Martín (1999). In low infections, the larvae of *T. zimbabwensis* were primarily found in the intercostal muscles whereas in high and

medium infections the muscles of the fore and hind limbs served as predilection sites. The altering of predilection patterns suggest that the larvae of *T. zimbabwensis* disperse from the small intestine and occupy predilection muscles closest to their release site, such as the intercostal pillars, first before occupying the leg musculature.

This study also revealed that newborn larvae will, as infection intensity is increased, disperse to muscles of the cranial and caudal regions in a relatively uniform pattern. These results are consistent with the findings of Wright *et al.* (1989) that hypothesized that the larvae of *Trichinella* will invade predilection muscles first.

Consistent with results reported by La Grange *et al*., (2013) the crocodiles in this study harboured more larvae in the superficial muscles of the dorsal aspect of the tail compared to the muscles located ventrally.

The longer time from infection to establishment of larvae in the muscles of crocodiles compared to mammalian hosts as shown by the results from biopsy samples collected on day 28 p.i. and day 60 p.i. of this study also support the findings of Pozio *et al*. (2004) that suggested that *T. zimbabwensis* larvae undergo a longer period of growth in reptiles.

Despite periodical fluctuations, mean blood glucose levels showed an overall increase in all three the experimental groups of this study and never reached levels below the normal values recorded on Day 0. This is in contrast with studies in mammals where hypoglycaemia induced by infection with *Trichinella* spp. larvae caused blood glucose to decrease below normal levels (Busila *et al*., 1968; Reina *et al*., 1989; Nishina & Suzuki, 2002; Wu *et al*., 2009). This would suggest a physiological adaptation in crocodiles similar to that reported in dolphins (Venn-Watson *et al*., 2011) allowing them to up-regulate glucose to meet extra demand of glucose when due. The variation in time from infection until minimum and maximum glucose levels were reached in the three experimental groups additionally suggest that larvae of *T. zimbabwensis* may be able to adjust their own growth rate according to the host metabolism and availability of nutrients.

Serum levels of CPK, LDH and AST have proven value as diagnostic procedures to detect human trichinellosis (Gottstein *et al*., 2009) and elevated levels of ALT in patients infected with *T. pseudospiralis* have also been reported in humans (Jongwutiwes *et al*., 1998) and in mammals (Wisniewska, 1970; Koudela & Schanzel, 1980; Soule *et al*., 1989; Ribicich *et al*., 2007 Mukaratirwa *et al*., 2008).

Mean ALT and AST concentrations of the experimental crocodiles on Day 0 were within the normal reference ranges for Nile crocodiles (Lovely *et al*., 2007; Botha, 2010) and in the case of ALT compared favourably with the ranges reported for American alligators (Diethelm & Stein, 2005). Mean CPK and LDH concentrations of the experimental crocodiles were within the normal reference ranges reported for Dwarf caimans (Diethelm & Stein, 2005) and CPK compared favourably with ranges reported for American alligators (Diethelm & Stein, 2005).

The kinetics of ALT concentrations of Nile crocodiles reported on in this study showed that individual host physiology supersede the effects of muscle damage caused by the larvae of *T. zimbabwensis.*

Peak levels of AST appeared late during the course of infection and changes in the ratio between ALT and AST were in agreement with reports in literature as indicators of muscle damage (Srivastava & Chosdol, 2007).

The kinetics of CPK indicates that peak levels coincided with the invasion of muscle tissue by the parasite larvae in contrast to the case in human trichinosis where increases in levels of this enzyme did not exceed the increases of other enzymes (Kocieska, 2000).

In contrast to studies in horses (Soule *et al*., 1989), peak concentrations of LDH in Nile crocodiles in this study did not coincide with peaks of CPK and ALT but were reached later in the course of the study. The kinetics of both LDH and AST observed in this study show that these enzymes may not necessarily be indicators of muscle damage, but rather appear to coincide with the migration of larvae. However, the results of this study support those observed in horses where LDH was found to be more sensitive to the effects of *Trichinella* infection than CPK.

Antibodies to *T. zimbabwensis* infection in crocodiles have previously been detected by indirect ELISA but the test was deemed unsuitable for surveillance purposes due to the unpersistent nature of the antibodies (Ludovisi *et al*., 2013). The literature shows that antibody titres cannot always be correlated with infection intensity (Pozio *et al*., 2002; Vu Thi *et al*., 2010; Ludovisi *et al*., 2013). Results from this study support these findings. The interpretation of antibody titres in crocodiles is also complicated as a result of the potential influence of age, temperature, season and hormone levels on the individual immune response of these animals (Brown *et al*., 2001; Ludovisi *et al*., 2013).

5.2 General Conclusion

1. Despite reports suggesting information to the contrary (Bruschi, 2012), human populations on the African continent are at considerable risk of infection from *Trichinella* spp. The situation may be exacerbated in as far as *T. zimbabwensis* is concerned due to the limited information on the epidemiology and distribution of *T. zimbabwensis* on the African continent.

2. The poor socio-economic development and lack of infrastructure prevalent in many African countries cannot realistically be improved in the foreseeable future and thus the impact of these factors, in as far as compounding the risk of infection is concerned, will in all probability remain a considerable obstacle in the future.

3. Numerous studies have been undertaken since the initial discovery of *T. zimbabwensis* in an attempt to gain a better understanding of its epidemiology and interactions with different host species. Although both experimental and natural studies involving crocodiles are precluded through their vast distribution (Botha, 2010), the dangers associated with working with crocodiles (Richardson *et al*., 2002; Huchzermeyer, 2003; McGregor, 2005; Botha, 2010) and conservation legislation (Pozio, 2005) a need exists for more studies specifically focussed on these predators and their interaction with *T. zimbabwensis*.

4. Results from this study additionally show unique distribution and predilection patterns for *T. zimbabwensis* in Nile crocodiles in the muscles of the fore and hind limbs as well as the intercostal muscles. These results are in contrast with recommendations for the use of masseter, pterygoid and intercostal muscles for the detection of *T. zimbabwensis* in crocodiles by the European Commission (2005).

5. Additionally, the findings of La Grange *et al*. (2013) for the use of biopsy samples for surveillance purposes are supported.

6. The contrasting effects of *T. zimbabwensis* infection on blood glucose levels and the observed kinetics of selected biochemical parameters of crocodiles in this study compared to results from studies in mammals (Busila *et al*., 1968; Reina *et al*., 1989; Soule *et al*., 1989; Nishina & Suzuki, 2002; Kociecka, 2000; Wu *et al*., 2009) further confirm the unique host-parasite relationship.

7. Results from this study confirm the unsuitability of ELISA testing in crocodiles as reported in literature (Ludovisi *et al*., *in press*).

8. The aforementioned not only confirms the existence of a need for more controlled, experimental and field studies to elucidate the epidemiology and distribution of *T. zimbabwensis,* but also to gain more insight into the normal physiological processes of the Nile crocodile that may influence its unique relationship with the parasite.

9. Veterinary and health authorities across the African continent should foster closer relationships and concerted efforts should be made to assess the actual potential risk of infection to human populations.

10. Effective control measures based on sound scientific knowledge should be instituted to protect populations at risk of infection.

5.3 References

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