

**THE USE OF AN INACTIVATED VACCINE IN FARMED
NILE CROCODILES (*CROCODYLUS NILOTICUS*) FOR
THE CONTROL OF *MYCOPLASMA CROCODYLI*
INFECTION**

By

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DEDICATION

“Not to us, Lord, not to us but to your name be the glory, because of your love and faithfulness.”

Psalm 115:1

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TABLE OF CONTENTS

Dedication	ii
Acknowledgements	iii
Table of Contents	iv
List of Tables	vii
List of Figures	viii
Summary	1
Chapter 1: Introduction	3
Chapter 2: Literature review	5
<i>M. crocodyli</i>	5
History of <i>Mycoplasma</i> outbreaks	5
Disease caused by <i>M. crocodyli</i>	5
Comparison between <i>M. crocodyli</i> and other pathogenic <i>Mycoplasma</i> spp	6
<i>Mycoplasma</i> spp infections of the respiratory tract	7
<i>Mycoplasma</i> spp infections causing arthritis	7
<i>Mycoplasma</i> infections of other reptiles	7
Crocodile husbandry and mycoplasmosis	10
General aspects of crocodile farming	10
Crocodilian stress factors	11
Impact of stress on crocodilians	11
Pathogenesis of mycoplasmosis	12
Intracellular location of <i>Mycoplasma</i> spp	12
Adhesion to host cells and antigenic variation	13
Interaction between <i>Mycoplasma</i> spp and the host immune system	14
Reptilian immune system	14
Adaptive immunity	15
Control of mycoplasmosis	16
Vaccination	16
Serodiagnosis of mycoplasmosis	18
Latex agglutination test	18
Metabolic inhibition assay	19

Chapter 3: Experimental design and methods.....	20
Facilities and experimental animals	20
Study subjects	20
Housing conditions	20
Nutrition, feeding and watering	20
Daily care of animals	21
Handling and restraint of crocodiles.....	21
Farm layout	21
Cleaning and disinfection	22
Biosecurity.....	22
Vaccine production.....	22
<i>Mycoplasma</i> -strain included in experimental vaccine.....	22
Vaccine production and formulation.....	23
Quality control of vaccine	23
Safety testing of the autogenous vaccine	23
Efficacy testing of the autogenous vaccine.....	23
Serum collection for serological testing	25
Sampling points for serum collection	25
Procedure for serum collection	26
Procedure for serum processing and transport to laboratories.....	26
Latex slide agglutination test.....	27
<i>Mycoplasma</i> strains and growth conditions	27
Coating of microspheres	27
Procedure for latex slide agglutination.....	27
Growth/metabolism inhibition assay	28
<i>Mycoplasma</i> strain and culture	28
<i>Mycoplasma</i> medium	28
Procedure for MI test.....	28
Statistical evaluation of results.....	28
Evaluating sero-conversion.....	29
Evaluating the association between serological status, disease status and vaccination status during disease outbreak.....	29

Evaluating the diagnostic performance of the latex agglutination test.....	29
Chapter 4: Results	30
Safety trial	30
Efficacy trial.....	30
Latex agglutination assays.....	30
Growth/metabolic inhibition assays.....	31
Results from outbreak, October 2011	32
Pathology	32
Serology	33
Statistical evaluation of results.....	34
Evaluating the relationship between vaccination and seroconversion.....	34
Evaluating the relationship between vaccination status, serological status and development of clinical disease during a disease outbreak	35
Evaluating the diagnostic performance of the latex agglutination test.....	36
Chapter 5: Discussion	37
Vaccine safety.....	37
Vaccine efficacy	37
Immunogenicity	37
Vaccine efficacy during a disease outbreak.....	39
Performance of the latex agglutination assay.....	44
Chapter 6: Conclusion.....	46
Reference List	47

LIST OF TABLES

Table number	Table title	Page number
Table 3.1	2x2 contingency table for determining diagnostic performance	21
Table 4.1	Summary of LAT results	31
Table 4.2	Summary of MI test results	31
Table 4.3	Summary of outbreak results - LAT	33
Table 4.4	Summary of outbreak results - MI test	34
Table 4.5	Relationship between LAT results and MI test results	36

LIST OF FIGURES

Figure number	Figure title	Page number
Figure 3.1	Layout of crocodile unit of Nuanetsi Ranch, Mwenezi, Zimbabwe	21
Figure 3.2	Schematic representation of trial	24
Figure 3.3	Vaccination of crocodiles.	25
Figure 3.4	Crocodile tail indicating the healed lesion after removal of one scute from the right	25
Figure 3.5	Collecting blood from a crocodile.	26
Figure 4.1	Layout of a typical LAT plate.	30
Figure 4.2	Two examples of positive reactions	30
Figure 4.3	An example of a negative reaction.	30
Figure 4.4	Acute arthritis.	32
Figure 4.5	Subacute arthritis.	32
Figure 4.6	Chronic arthritis.	33
Figure 4.7	Typical skin lesions on the feet and over the sternum	33
Figure 4.8	Percentage seropositive animals at different times after vaccination	34
Figure 4.9	Percentage of crocodiles in each LAT titre range	35
Figure 4.10	Percentage of crocodiles in each MI assay titre range	36

SUMMARY

THE USE OF AN INACTIVATED VACCINE IN FARMED NILE CROCODILES (*CROCODYLUS NILOTICUS*) FOR THE CONTROL OF *MYCOPLASMA CROCODYLI* INFECTION

Since the first report of *Mycoplasma*-associated polyarthritis in farmed Nile crocodiles in 1995, the disease has spread across Zimbabwe and South Africa and has resulted in significant economic losses on infected farms. Due to poor response to antimicrobial treatment and frequent relapses, the use of an autogenous vaccine to manage disease outbreaks was evaluated. Two previous trials had been performed with a similar vaccine and the results suggested that the vaccine could be effective in alleviating disease, although the numbers of animals were limited in both. This trial aimed to evaluate an inactivated, alum-adjuvanted *M. crocodyli* whole-cell vaccine in a large group of yearling crocodiles under field conditions on a farm in Zimbabwe where repeated *M. crocodyli* outbreaks have been reported.

The safety of the vaccine was assessed by administering the vaccine intraperitoneally to a subset of crocodiles. No adverse clinical reactions were observed in any of these crocodiles.

A group of two thousand two hundred crocodiles received two intramuscular vaccinations four weeks apart in the autumn of 2011, while another group of two thousand two hundred crocodiles served as unvaccinated controls. Serum was collected from a subset of the vaccinated and unvaccinated crocodiles at different time-points before and after vaccination to evaluate the humoral response to vaccination. Latex slide agglutination tests (LAT) were performed on all samples and positive samples were titrated with the latex slide agglutination test and metabolism inhibition assay.

A low percentage of sera were positive with serological tests done prior to vaccination, suggesting either circulating *Mycoplasma* or maternal immunity. Statistically significant increase in sero-positivity was detected with LAT four weeks after primary vaccination, although the titre remained low. Six weeks after the booster vaccination the percentage seropositive vaccinated crocodiles had decreased and there were no statistically significant difference between the percentage seropositive vaccinated and unvaccinated crocodiles.

A significant outbreak of *Mycoplasma*-like polyarthritis was encountered 6 months after vaccination, in October 2011. Both vaccinated and unvaccinated crocodiles were affected. Serum samples from different subsets of crocodiles were collected and evaluated similar to the vaccine trial. The results indicated that a similar rate of sero-positivity was present in all crocodiles, irrespective of vaccination- or disease status.

Sera collected during this trial was used to evaluate the performance of the latex slide agglutination assay compared to the metabolism inhibition assay ("Gold standard" assay), as the performance of the LAT had not been evaluated previously. The calculated diagnostic sensitivity was 72%, diagnostic

specificity was 32%, the predictive value of the positive test was 36% while the predictive value of the negative test was 69%.

This trial indicated that the autogenous, inactivated, alum-adjuvanted, whole-cell vaccine against *M. crocodyli* was not able to protect farmed Nile crocodiles on an infected farm against clinical *Mycoplasma*-associated polyarthritis. It was also found that the latex slide agglutination assay could be useful as a robust, pen-side assay to evaluate exposure to *M. crocodyli*, although other assays, such as PCR, bacterial culture or growth inhibition assays, has to be performed to confirm the presence of disease.

CHAPTER 1: INTRODUCTION

Farming of crocodylians is primarily concerned with the production of crocodile skins for luxury leather markets. Although this is a fluctuating market, it is estimated that between one and two million crocodylian skins are internationally traded annually (Caldwell 2012). The brown caiman (*Caiman crocodilus fuscus*) is the “top-seller” and accounts for around half of exported skins, followed by the American alligator (*Alligatoris mississippiensis*) and the Nile crocodile (*Crocodylus niloticus*) (Caldwell 2012). Crocodile meat is also internationally traded but is regarded as a by-product of skin production (Caldwell 2010).

Zimbabwe is the largest producer of Nile crocodile skins with approximately half the annual CITES-reported Nile crocodile skins exported from that country. Commercial trade in crocodile skins has been a key driving factor in the rescue of the species in Zimbabwe, because of the economic value that is currently attached to this species that was previously classified as vermin and hunted almost to extinction (Revel 1995, Caldwell 2010). Since the first crocodile farm was established in the mid-1960s, the production of skins has progressively shifted from wild-harvested skins to captive-bred skins (Caldwell 2010). With a proportion of bred crocodiles re-introduced into the wild, the Zimbabwean wild population (as well as the wild populations in most of Southern African countries) is currently listed under Appendix II of CITES (Ferguson 2010).

Crocodile farming, particularly in Zimbabwe, also has the benefit of creating employment and socio-economic improvement in this economically challenged country. Nuanetsi crocodile ranch, Mwenezi, Zimbabwe is a good example of such a project. Despite the political controversy surrounding the farm (Scoones *et al.* 2012), more than 2000 employment opportunities have been created (Riley 2010).

Recurrent epidemics of polyarthritis and paralysis were reported on Nuanetsi in 2010, affecting up to 40% of rearing stock as well as breeding stock. Financial losses of around \$1 million were experienced, severely hampering the economic sustainability of the operation. During August 2010, South African researchers and crocodile experts were approached to assist with the investigation and management of these outbreaks. *Mycoplasma crocodyli* was isolated from arthritic lesions in affected crocodiles and confirmed as the causative agent.

Mycoplasmosis in farmed Nile crocodiles is clinically characterized by polyarthritis of the appendicular and axial skeleton. Crocodiles consequently become lame, paretic and paralytic, fail to feed and starve to death. Paralysis is the most common sign reported by commercial farmers. Overgrowth of normal commensal bacteria and fungi on the skin of paralysed crocodiles result in the development of ulcers and scars, which further reduces hide value, thereby worsening the economic effects of the disease.

In response to the mentioned outbreak, the managers of Nuanetsi Ranch and the scientists and veterinarians involved, decided to develop an experimental, autogenous vaccine against the cultured

organism. This route was decided on because the current method of control on commercial farms relies on the application of antimicrobial therapy, which is costly and did not provide the expected clinical improvement during outbreaks. A similar vaccine had been developed and tested on two previous occasions in Zimbabwe (Mohan *et al.* 1997, Mohan *et al.* 2001), and, although some of the results were promising, the vaccine had not previously been tested in a large population in the face of a disease outbreak.

The primary objectives of the study were therefore, to test and demonstrate the safety and efficacy of an experimental, inactivated, alum-adjuvanted *Mycoplasma crocodyli* whole-cell vaccine in a large group of yearling crocodiles under field conditions on a farm in Zimbabwe where repeated *M. crocodyli* outbreaks have been reported. A secondary objective arose from the serological tests, namely to evaluate the diagnostic performance of a latex slide agglutination assay which had been developed by the same researchers.

CHAPTER 2: LITERATURE REVIEW

In this section, the relevant literature on *M. crocodyli* will be considered and important aspects highlighted. As mentioned in the previous chapter, vaccination against *M. crocodyli* has been evaluated on two previous occasions. Because *M. crocodyli* is a relatively recently described pathogen, aspects of other *Mycoplasma* species will also be included where applicable. As *Mycoplasma* infections in most species are encountered in intensive housing and production setups, relevant management and stress factors will be considered. Reptile immunology is also of importance, as vaccination and sero-diagnostics cannot be contemplated without understanding the host immune response to a pathogen. Lastly, vaccination and sero-diagnosis (the focus of this study) will be reported on.

M. crocodyli

History of Mycoplasma-outbreaks

The first published outbreak of *Mycoplasma* associated disease in crocodylians was reported in Zimbabwe in 1995 (Mohan *et al.* 1995). Since this first outbreak, the disease has reportedly spread across the country, with approximately 35% of commercial farms affected by 2001. It has also been diagnosed in South Africa, where it is reported to be widespread (Huchzermeyer & Picard 2004), the Canary Islands and Israel (Huchzermeyer *et al.* 1997). A similar disease has also been reported in alligators in the USA, but with a dramatically higher mortality rate than described for *M. crocodyli* outbreaks (Clippenger *et al.* 2000).

The first outbreak affected only young crocodiles (1-3 years), and low morbidity and mortality were reported (Mohan *et al.* 1995). Low morbidity and mortality have also been reported for South African outbreaks thus far (F.W. Huchzermeyer, unpublished results, 2011). A significantly higher morbidity and mortality rate was however, encountered during a second published outbreak in Zimbabwe (Mohan *et al.* 2001). More than 2500 crocodiles were affected and morbidity peaked at 50% while mortality was estimated at over 20% (Mohan *et al.* 2001). It was suggested that this outbreak was triggered by translocation stress (Mohan *et al.* 2001).

Disease caused by M. crocodyli

Soon after the first outbreak, *Mycoplasma crocodyli* was named, classified and described as a new *Mycoplasma* species (Kirchhoff *et al.* 1997). *M. crocodyli*, as with other *Mycoplasmas*, lacks true cell walls and has a typical fried-egg appearance on solid medium, but grows relatively well in artificial medium (Kirchhoff *et al.* 1997). Glucose and mannose are both fermented, and cholesterol or serum is required for growth (Kirchhoff *et al.* 1997). It is one of the few *Mycoplasma* spp that fulfils Koch's postulates for disease causation (Kirchhoff *et al.* 1997). *M. crocodyli* has a peculiar preferred temperature range for *in vitro* growth as optimal growth is described at 37 °C a temperature which could be lethal for its host (Kirchhoff *et al.* 1997, Huchzermeyer 2002), while it would be expected that a pathogen of an exothermic host would prefer temperatures closer to the host's natural temperature range (Razin 2006). It is unknown whether this temperature preference holds true for *in vivo*

conditions. *Mycoplasma crocodyli* has a low G+C content (27.6%) which is characteristic for *Mycoplasmas* (Kirchhoff *et al.* 1997).

The complete genome sequence of *M. crocodyli* has recently been reported but, although at least five potential virulence factors have been identified, their role and significance are still unclear, particularly as no acknowledged adhesion factors have been identified (Brown *et al.* 2011).

Polyarthritis is the best described clinical and pathological sign associated with *M. crocodyli* (Mohan *et al.* 1995). Clinical signs of polyarthritis include progressive weakness, ranging from stiffness to complete immobility (Mohan *et al.* 1995). Both the appendicular and axial skeletons are affected and joints display marked swelling, although this may be difficult to appreciate ante-mortally in the spinal column (Mohan *et al.* 1995). Different stages of exudative polyarthritis are encountered at necropsy, ranging from turbid mucous containing *Mycoplasma* spp in acute and subacute cases, to yellow, inspissated exudate in chronic cases (Mohan *et al.* 1995). Histopathological changes include inflammatory oedema of the surrounding tissue, necrosis of the superficial layers of the synovial membrane, and fibrin deposition, lymphocytic infiltration and fibrosis of the joint capsule (Mohan *et al.* 1995). Joint fluid and heart blood are good samples for the culture of the organism.

Apart from polyarthritis, the organism also triggers pneumonia, histopathologically characterised by consolidation and oedema of affected areas, with a white blood cell (particularly polymorphonuclear cells and mononuclear cells) and erythrocyte infiltration (Mohan *et al.* 1995). Although the respiratory involvement of *M. crocodyli* is less often recognized, the respiratory tract is a common predilection site for *Mycoplasma* spp. colonization in many hosts and is the likely site for host invasion.

Comparison between *M. crocodyli* and other pathogenic *Mycoplasma* spp

Mycoplasma spp are some of the most widespread parasites of living organisms, and have been found in association with mammals, reptiles, birds, fish, arthropods and plants (Razin 1998). Over 200 species have been identified to date (Chazel *et al.* 2010), and it has been suggested that this is but a fraction of existing species (Razin & Hayflick 2010). Although only a small proportion of these are regarded as pathogenic, a range of conditions of animals and humans is associated with *Mycoplasma* spp. These include respiratory disease, mastitis, keratoconjunctivitis, arthritis and synovitis, as well as reproductive disorders and infectious anaemia. Little is known about *Mycoplasma* spp of crocodiles and vaccination against reptilian mycoplasmosis in general. This section will briefly mention pathogenic *Mycoplasma*-infections to which crocodile *Mycoplasma* can be related (primarily respiratory and joint complications) before considering mycoplasmosis of other reptiles. The successes and failures of vaccines (particularly inactivated vaccines) and sero-diagnostic tests against some of these mycoplasmas will be considered later in this chapter.

Mycoplasma spp infections of the respiratory tract

Colonization and infection of the respiratory tract is the best-described *Mycoplasma* spp pathology and two disease syndromes can be differentiated. The first is characterized by subacute to chronic interstitial pneumonia and/or bronchopneumonia, with or without non-specific upper respiratory disease. In this instance, infection with *Mycoplasma* spp. seldom results in fulminant disease by itself. It increases the hosts' susceptibility to other pulmonary pathogens, particularly bacteria, resulting in bacterial bronchopneumonia, which often masks the *Mycoplasma* infection (Ley 2006, Caswell & Archambault 2008, Sibila *et al.* 2009). Well-known *Mycoplasma* spp associated with this syndrome include *M. pneumoniae* (humans), *M. gallisepticum* (poultry), *M. hyopneumoniae* (swine), *M. bovis* (cattle) and *M. ovipneumoniae* (sheep).

Contagious pleuro-pneumonia is the second important respiratory complication associated with *Mycoplasma* spp colonization of the respiratory tract. Contagious bovine pleuro-pneumonia (CBPP), caused by *M. mycoides* subsp. *mycoides* Small colony, and contagious caprine pleuro-pneumoniae (CCPP), caused by *M. capricolum* subsp. *capripneumoniae*, are examples of this condition. Both are classified as diseases of major economic importance by the OIE (World Organisation for Animal Health), with CBPP being recognized as the most important transboundary disease of cattle in Africa (Nicholas & Churchward 2012, Thiaucourt *et al.* 2012). CBPP and CCPP differ from other respiratory mycoplasmoses, as it can cause fatal disease by itself with prominent involvement of the pleural membranes and pleural effusion (Thiaucourt 2004, Nicholas *et al.* 2008). Macroscopic sequestra are often encountered in recovered chronically infected animals (Thiaucourt 2004), and serve as the source of infection for other hosts.

Although some fatal cases of *M. crocodyli* disease have been described (Mohan *et al.* 2001), the general pulmonary pathology is more consistent with what is described for *M. bovis*, *M. gallisepticum* and *M. hyopneumoniae*, i.e. chronic pulmonary infection/colonization and inflammation.

Mycoplasma spp infections causing arthritis

Polyarthritis is often the main lesion associated with *M. crocodyli*. It is believed to result from the systemic spread of the organism, which has a tropism for serous membranes, such as pleura, pericardium and synovial membranes. Well-described *Mycoplasma*-associated arthritis agents include *M. synoviae* (poultry), *M. hyosynoviae* (swine), *M. bovis* (cattle), *M. agalaciae* (small ruminants) and *M. arthritidis* (rodents). The pathological lesions described for *M. crocodyli* are similar to the joint pathology described for most of the mentioned diseases (Hagedorn-Olsen *et al.* 1999, Kleven 2006, Nicholas *et al.* 2008, Hewicker-Trautwein *et al.* 2009).

Mycoplasma infections of other reptiles

As mentioned above, *Mycoplasma* spp parasites have also been identified in reptiles other than crocodiles. A summary of identified *Mycoplasma* spp, their host range and associated disease

syndromes is provided in Table 2.1 below. Some important characteristics of the two best described reptile *Mycoplasma* spp, namely *M. alligatoris* and *M. agassizii*, will be considered in more detail.

Table 2.1: ***Mycoplasma* species isolated from reptiles**

<i>Mycoplasma</i> species	Host	Effect of colonization
<i>Mycoplasma agassizii</i>	Tortoise (<i>Gopherus</i> sp & <i>Testudo</i> sp) and Turtles (<i>Terropene carolina</i>)	Chronic upper respiratory tract disease
<i>Mycoplasma testudineum</i>	Tortoise (<i>Gopherus</i> spp)	Chronic upper respiratory tract disease
<i>Mycoplasma alligatoris</i>	Alligators (<i>Alligator mississippiensis</i>) and caimans (<i>Caiman latirostris</i>)	Acute multisystemic inflammatory disease
<i>Mycoplasma crocodyli</i>	Crocodiles (<i>Crocodylus niloticus</i>)	Polyarthritis, sub-acute pneumonia
<i>Mycoplasma testudinis</i>	Tortoise (<i>Testudo graeca</i>)	Commensal
Unnamed <i>Mycoplasma</i>	Burmese python (<i>Python molurus bivittatus</i>)	Proliferative tracheitis and pneumonia
<i>Mycoplasma iguanae</i>	Green iguana (<i>Iguana iguana</i>)	Vertebral disease

MYCOPLASMA ALLIGATORIS INFECTION OF AMERICAN ALLIGATORS (*ALLIGATOR MISSISSIPPIENSIS*)

M. alligatoris causes fatal multisystemic inflammatory disease in American alligators characterized by pneumonia, fibrinous polyserositis and polyarthritis (Clippenger *et al.* 2000, Brown *et al.* 2001b). Although *M. alligatoris* has been proven a distinct species, it is closely related to *M. crocodyli*, with only a 2% difference in the 16S rRNA sequence (Brown *et al.* 2001a). Infection caused by *M. alligatoris* however, progresses faster and is more virulent than *M. crocodyli*-associated infection (Brown *et al.* 2011). The reasons for the increased virulence have been investigated, but despite the identification of potential virulence genes and “spreading factors”, more research is required before this phenomenon can definitively be explained (Brown *et al.* 2004, Hunt & Brown 2005, Hunt & Brown 2007, Brown *et al.* 2011).

It has been suggested that *M. alligatoris* infects a wider host range than American alligators. When administered under experimental conditions, fatal disease could be induced by *M. alligatoris* in closely related broad-nosed caimans (*Caiman latirostris*) while more distantly related Siamese crocodiles (*Crocodylus siamensis*) seroconverted (Pye *et al.* 2002). There have also been reports that *M. alligatoris* is responsible for some of the *Mycoplasma*-problems on Nile crocodile farms in South Africa (F.W. Huchzermeyer, personal communication, 2011). From this information, it can be suggested that

the epidemiological investigation of *M. crocodyli*, particularly with regards to reservoir hosts, might require sampling and testing of other reptile species as well.

MYCOPLASMOSIS IN TORTOISES

Upper respiratory tract disease (URTD) is a disease syndrome of various tortoise species (including gopher tortoise (*Gopherus polyphemus*) and desert tortoise (*Gopherus agassizii*)), currently ascribed to *Mycoplasma agassizii* and *M. testiduneum* infection (Brown *et al.* 1994, Brown *et al.* 1999, Wendland *et al.* 2005). Other microorganisms may also be involved (Sandmeier *et al.* 2009). The main clinical signs are chronic rhinitis and conjunctivitis, resulting in serous to mucopurulent nasal and ocular discharge with partial or complete occlusion of one or both nares (Jacobson *et al.* 1991, Lederle *et al.* 1997, Homer *et al.* 1998, McLaughlin *et al.* 2000, Christopher *et al.* 2003, Wendland *et al.* 2005). Systemic effects are non-specific and include generalized cachexia and lymphocytic infiltration (Wendland *et al.* 2005). A fatal outcome of disease is associated with secondary infections and/or nutritional and metabolic disturbances (Jacobson *et al.* 1991, Homer *et al.* 1998, McLaughlin *et al.* 2000).

Mycoplasmosis of tortoises do not resemble mycoplasmosis of crocodiles, but some aspects of the sero-diagnostics will be described as it emphasizes the importance of critical analysis of serological tests and their results in general, but also specifically for reptile mycoplasmosis.

Because URTD was implicated as a causative factor for a decline in desert tortoise populations in the 1980s and 1990s, a conservation plan was formulated according to which all ELISA positive or suspect -positive tortoises, destined for translocation, should be euthanized (Brown *et al.* 1994, Schumacher *et al.* 1997, Homer *et al.* 1998, Seigel *et al.* 2003, Sandmeier *et al.* 2009). The plan aimed to limit/prevent the spread of the disease to other tortoise populations and was based on the positive correlation described between clinical signs and ELISA seropositivity (Schumacher *et al.* 1993, Schumacher *et al.* 1997).

This strategy was recently challenged, primarily because of the demonstration of natural antibodies to *M. agassizii* in desert tortoises but also due to problems with the ELISA (Hunter *et al.* 2008, Sandmeier *et al.* 2009). Natural antibodies (described in more detail under the section on reptile immunology) is of note as the titres, which are influenced by individual variation and not disease exposure, could be high enough to be recorded as positive (Sandmeier *et al.* 2009). Unexposed animals could thus be classified as seropositive, and handled accordingly. Problems with the ELISA include the absence of a gold standard assay, monoclonal antispecies conjugate against only one type of chelonian immunoglobulin light chain and variability in ELISA cut-off values (Sandmeier *et al.* 2009). The prescribed management strategy regarding a positive ELISA result from an individual tortoise as an indication of a shedder of the organism, is also problematic. This is in contrast to the more common interpretation that a single sero-positive ELISA only indicates current infection if persistent infection have been proven for the specific disease.

Apart from demonstrating the potential pitfalls of serodiagnostics, the problems also emphasize how the current gaps in our understanding of the reptilian immune system complicate the interpretation of diagnostic assays that were developed for mammalian hosts.

Crocodile husbandry and mycoplasmosis

A clear pattern for husbandry practices, associated with mycoplasmosis can also be identified for crocodiles, namely that it is persistently associated with intensive production systems (Bradbury 2005). This is similar to the recurring pattern of host tissue tropism described above. Several characteristics of these systems enhance the general likelihood of infection, including close contact between animals (particularly for environmentally sensitive pathogens such as *Mycoplasma* spp and viruses), frequent addition of immunologically naïve animals, and increased host stress (social stress due to high stocking densities, metabolic stress due to abnormal feeding practices, environmental stress due to temperature extremes etc.) (Nicholas & Ayling 2003). For various pathogens (including *M. gallisepticum* (Bradbury 2005)), it has been shown that the interaction of various environmental factors with the organism, results in the potentiation of a previously imperceptible disease into one of economic importance. Therefore, when considering the epidemiology and control strategies of *Mycoplasma* spp, it is unavoidable to consider the environmental factors involved in the disease, and particularly those factors that could influence the host immune response.

General aspects of crocodile farming

Crocodiles are farmed for their skins, which are used in the production of luxury leather goods. Most crocodile farms consist of various operations, including a hatchery, rearing facilities, abattoir, feed mixing and storage, and a breeding colony. The typical production processes include seasonal collection of eggs from the breeders, followed by artificial incubation and the raising of the young from hatching until a suitable size has been reached. Skins “harvesting” is performed in accordance to the size preference of the leather industry.

Rearing can be performed indoors or outdoors. Pens are lined with concrete and typically include pond and dry areas. Pen walls should be constructed high enough to prevent escape of crocodiles. On some farms, shade and/or heating may be provided. An ideal stocking density for pens has been suggested by Huchzermeyer (2003) but it is unlikely that this guideline is followed on all farms because of the expense of the construction of concrete pens.

Various feeding strategies are followed. Many farms include animal carcasses in feed, at least to breeder crocodiles. Some farms may feed predominantly animal protein (carcasses) while others feed a formulated ration, which include carbohydrates, fats, minerals and vitamins. Feeding intervals vary from farm to farm; although it is suggested that gastric emptying takes 36 hours in crocodiles, many farms perform daily feeding (Huchzermeyer 2003).

Cleaning of pens, with high pressure hoses, should be performed at least daily and it is suggested that a detergent is applied on a weekly basis. This is due to the high levels of bacteria present in crocodilian faeces and the build-up of a layer of fat in the pens, due to leached-out and undigested fat (Huchzermeyer 2003).

Guidelines for keeping crocodiles have been published (Huchzermeyer 2003, Peuker *et al.* 2005) and the importance of keeping crocodiles stress- and disease-free and well-nourished has been stated, but not all the guidelines are necessarily followed and several stress factors have been identified. The most important of these factors are discussed below, followed by a brief discussion on the influence of stress on the host.

Crocodilian stress factors

Environmental temperature is one of the most important stress factors for poikilothermic animals such as reptiles. Overheating and chilling could both cause stress in crocodiles. Under commercial crocodile housing conditions in temperate regions both could result as different thermal environments are seldom provided (Huchzermeyer *et al.* 2002). Concrete crocodile housing often does not provide shade or shelter, and ponds are relatively shallow, not protected from the sun and do not have a constant inflow of water. Therefore, crocodiles cannot make use of thermal gradients to maintain ideal body temperature because these are not available, and the animals are particularly vulnerable to environmental temperature fluctuations.

A second common stressor is the handling of crocodiles. As could be expected for a non-domesticated species, capture and restraint are stressful as it neutralises the crocodile's natural flight instinct (Huchzermeyer 2003). Scientific studies on various methods of restraint detected a significant increase in various blood parameters, including corticosterone, in estuarine crocodiles (*Crocodylus porosus*) following manual restraint (Franklin *et al.* 2003). Capture and restraint are performed for various reasons on crocodile farms, including the movement of animals, measurement and skin inspection and teeth trimming.

Overstocking is another relatively common stressor of captive animals. Stress results as overstocking prevents crocodiles from moving away from other individuals when threatened, which is a natural instinct (Huchzermeyer 2003). The positive link between stocking density and plasma corticosteroid levels in captive alligators has been confirmed experimentally (Elsey *et al.* 1990).

Impact of stress on crocodilians

Environmental stress factors are discussed above and, together with other management factors, such as abnormal social groups and procedures such as electro-stunning (Cash *et al.* 1997, Huchzermeyer 2003, Morgan & Tromberg 2007), it can cause acute and chronic stress. Chronic stress is of particular importance as glucocorticoids have a significant effect on the host immune system (Dickens *et al.* 2010).

As for most species, long term increased glucocorticoid levels are immunosuppressive to reptiles (Saad *et al.* 1986, Huchzermeyer 2003). Suppression of macrophages, T-lymphocytes and plasma cells have been reported in various reptilian species (Saad *et al.* 1984, Saad *et al.* 1986, Mondal & Rai 2002, Hareramadas *et al.* 2004), although the molecular regulation has not been detailed (Verbrugge *et al.* 2011).

A second important consequence of stressful events to crocodiles is the disruption of the gut mucosal barrier, which results in the translocation of gut commensal bacteria into the systemic circulation, resulting in septicaemia (Huchzermeyer 2003). Under normal circumstances, these organisms will be removed by the host immune system, but if host immunity is impaired (as a result of stress), systemic invasion and pathology could result (Huchzermeyer 2003).

In conclusion, it can be stated that, in contrast to the common misconception that reptiles are quite tolerant to abnormal conditions (Case *et al.* 2005), it is clear that stress is also experienced and can have a profound influence on the health and welfare of reptiles. Several common husbandry practices could lead to stress and subsequent immune suppression. Immune suppression not only influences the ability of an animal to eliminate a potential pathogenic infection, but also influences the immune response to vaccination.

The following section will deal with the environmental factors that play a role in the outcome of infection, followed by an examination of some of the mechanisms employed by the pathogen (*Mycoplasma* spp) in the development of disease.

Pathogenesis of mycoplasmosis

Mycoplasma spp have an exceptionally wide host range and tropism for a variety of tissues. *Mycoplasma* spp have been described as perfect parasites as the majority occur as commensals in their hosts without causing any harm (Razin & Hayflick 2010). The reasons for and the pathogenesis of disease caused by pathogenic *Mycoplasma* spp are still under investigation, although molecular studies on these organisms have illuminated some interesting facts, including the importance of the interaction between the organisms and host cells (i.e. adhesion to host cells and/or intracellular location of some *Mycoplasma* spp), the expression of surface-antigen variation, and the modulation of the host immune system by *Mycoplasma* spp (Razin 2006). All these aspects could play a role in the host immune reaction to the pathogen and , the efficacy of the host immune reaction in eliminating the microorganism and, therefore, in the efficacy of vaccination as a control strategy. Recent discoveries in these areas are thus reviewed in this section.

Intracellular location of Mycoplasma spp

Since the report of the intracellular location of *M. penetrans* (Lo *et al.* 1993), the invasion of host cells by *Mycoplasmas* has been reported for various pathogens including *M. pneumoniae* (Yovlavich *et al.*

2004), *M. fermentans*, *M. genitalium* (Rottem 2003) and *M. gallisepticum* (Winner *et al.* 2000). The mechanism/s by which these organisms gain entry is not well understood, but from what is known, it seems that various species make use of different approaches (Rottem 2003).

The most important consequence of the intracellular location of the *Mycoplasma* is that this location will protect the organism from the host immune response and, even if only temporarily for a specific individual cell, will enhance the survival of the population and the chronicity of the infection (Razin 2006).

Adhesion to host cells and antigenic variation

It is accepted that the vast majority of *Mycoplasma* spp are extracellular pathogens that adhere tightly and persistently to host cells, particularly mucous epithelial linings despite the intracellular penetration that has been described for some (Razin 2006). Adhesion is recognized as a prerequisite for host colonization and infection (Razin 2006).

Mycoplasmal adhesins (membrane proteins and lipoproteins) are recognized as key role players in adhesion, although it is suspected that the process is multifactorial and involves accessory membrane proteins (Razin 2006). Adhesins, because of their position on the interface between the host and organism, and the cardinal role of adhesion in host colonization and infection, are also major targets of the host immune response (Citti *et al.* 2010).

Mycoplasmas cause chronic infections in immune-competent hosts, even in the face of an adaptive immune response, despite the fact that it would be expected that *Mycoplasmas*, with their reduced genomes, lack of sophisticated genetic machinery to evade the host immune system, and lacking a rigid cell wall would be removed from the host relatively easily (Razin 2006). The discovery of phase and antigenic variation has provided an explanation for this discrepancy.

Antigen and phase variation refer to the genetic events, which lead to phenotypic changes in the structure and composition of adhesins and other major surface antigens (Citti *et al.* 2010). These events are reversible, mutation-based and result in a phenotypic heterogeneous population in which certain cells are capable of surviving despite environmental challenges, particularly the host immune response (Razin 1998, Citti *et al.* 2010). Since the first description of phase variation in 1990 (Rosengarten & Wise 1990), substantial research effort has gone into the investigation of antigenic variation in various pathogenic *Mycoplasma* spp (Citti *et al.* 2010). It has become clear that the presence of antigenic variation is wide spread among *Mycoplasma* spp but has evolved independently in different species (Razin 2006). The described mechanisms include mechanisms for ON/OFF switching of genes or combinations of genes (phase variation), variation in the size of antigens (size variation) (based on repeating certain regions for a variable number of times) as well as domain shuffling, all which occur at a relatively high frequency (Citti *et al.* 2010).

A variation in surface antigens is an important feature in the persistence of *Mycoplasma* spp in the face of the host immune reaction as it presents the immune system with a constantly varying array of antigens. This has major implications for the development of vaccines, as vaccines will have to mimic this variation in order to stimulate complete protection against the pathogen (Citti *et al.* 2010).

Interaction between Mycoplasma spp and the host immune system

A complex interaction between *Mycoplasma* spp and their hosts has been described, as would be expected for a pathogen with sophisticated machinery to evade the host immune system. The host employs various specific protective mechanisms to eliminate the organism, including the production of systemic and local immunoglobulins of various classes, the stimulation of cell-mediated immune reactions and opsonisation and phagocytosis of invading cells (Razin 1998). *Mycoplasmas* on the other hand, have various mechanisms of resisting the host immune reaction (including the antigenic variation described above) and have been shown to not only suppress and/or modulate the host immune response (Muneta *et al.* 2008), but also play a role in development and exacerbation of lesions caused by *Mycoplasmas* (Razin 1998, Rottem 2003, Razin 2006).

A major implication of this complex interaction for the control of mycoplasmosis, particularly for vaccination, is that the stimulated immune response that is meant to protect the host against the disease, could in fact enhance disease severity. This has been described in some *Mycoplasma* spp vaccine trials where vaccinated animals developed more severe clinical and pathological signs (Bryson *et al.* 2002, Maunsell *et al.* 2009). Thus, the evaluation and characterization of both the immune-stimulatory and immune-pathological features of *Mycoplasma* spp seem obligatory in the development of effective disease management strategies.

Both previous sections have referred to the role of host immunity in the outcome of infection, particularly for *Mycoplasma* spp. This system is examined in the next section.

Reptilian immune system

There are still lacunas in our understanding of the reptilian immune system. Since the 1980s, significant research effort has gone into mammalian (particularly human) and avian immunology, while the interest in reptilian immunology has waned (Origgi 2007). This vacuum is particularly evident when species-specific knowledge is sought. General references on reptile immunology are present in the literature (see Origgi 2007 and Zimmerman *et al.* 2010) and therefore, this discussion has been shortened to focus on the adaptive immune system. It was felt that this system is important as (1) the two key characteristics of adaptive immunity (namely specificity to antigens and immunological memory) are fundamental in the practice of vaccination and (2) serology is based on the detection of immunoglobulins (humoral immune factors) in peripheral circulation.

Adaptive immunity

Immunoglobulins (antibodies) (Ig) form the humoral arm of the adaptive immune system while cell-mediated immunity constitutes the cellular arm. The key components of cell-mediated immunity are cytotoxic T-lymphocytes (and their helper-T lymphocytes) and the focus is intracellular antigens, while the main components of humoral immunity are Ig secreted by activated B-lymphocytes (called plasma cells). It has been shown that both B- and T-cells are present in reptiles (Coe 1972, Coe *et al.* 1976, Cuchens & Clem 1979, El Deeb 1990, Work *et al.* 2000, Burnham *et al.* 2005), although the mode of interaction between T- and B-cells needs clarification (Zimmerman *et al.* 2010).

Immunoglobulins have also been reported for a variety of reptiles (Coe 1972, Coe *et al.* 1976, Warr *et al.* 1995, Work *et al.* 2000, Origgi 2001). While five classes have been described in mammals (IgM, IgG, IgA, IgD and IgE) and four in birds (IgY, IgM, IgA and IgD), it has been demonstrated that most reptiles produce IgM and IgY, with evidence of IgD and IgA in some species (Zimmerman *et al.* 2010). Current research suggests that crocodylians have only IgM and IgY-like immunoglobulins (Origgi 2007).

Natural antibodies (Nabs) are also encountered (Longenecker & Mosmann 1980), but their role in reptile immunity has not been defined. These have been described in many different taxa from sharks to humans (Adelman *et al.* 2004). IgM-, IgA- and IgG-like Nabs have been described, although IgM seems to be the predominant isotype (Boes *et al.* 1998). They differ from “traditional” antibodies in that they are released spontaneously in the absence of specific antigen stimulation from B-cells that have a low antigen affinity but are polyreactive (Ochsenbein & Zinkernagel 2000). In essence, they function as part of the innate immune system (although they are produced by B-cells) by non-specifically targeting broad categories of antigens, such as bacteria and viruses (Boes *et al.* 1998, Ochsenbein & Zinkernagel 2000, Madsen *et al.* 2006). Nabs are often dismissed as non-specific background signals when serological assays are performed (Madsen *et al.* 2006). It is possible however, that these antibodies form an important part of the reptile immune system (Madsen *et al.* 2006, Zimmerman *et al.* 2010).

Significant differences in the kinetics of the antibody response and the timing of class switching in reptiles, as opposed to mammals, have been documented (Origgi 2007, Zimmerman *et al.* 2010). It is proposed that IgM only peaks 6 weeks after exposure and may still be detectable more than 20 weeks after an insult (Zimmerman *et al.* 2010). Furthermore, although it is suggested that antibody response after a second exposure is faster, it is stated that the isotype of the secondary response has not been determined (Zimmerman *et al.* 2010). These factors could have major implications for the development of serological assays aimed at the detection of certain antibody isotypes.

Immunological memory and antigen specificity are critical in the development of successful vaccines with class switching, somatic hypermutation and affinity maturation of immunoglobulins constituting the three cornerstones of increased antigen specificity (Origgi 2007). However, literature on these

characteristics in reptiles is contradictory, with some authors reporting negative results (Grey 1963, Turchin & Hsu 1996, Hsu 1998), while others report positive results (Coe 1972, Coe *et al.* 1976, Brown 2002). References to an anamnestic response suggest that immunological memory should be present in reptiles (Work *et al.* 2000, Huchzermeyer 2003).

In conclusion, it can be stated that there is a definite need for further research in reptile immunology, particularly in different classes, and that vaccination regimens and serological techniques deduced from mammalian and avian medicine should be interpreted with caution.

Control of mycoplasmosis

In general, mycoplasmosis control can be divided into three important sectors, namely vaccination, medication and keeping disease-free animals (Desrosiers 2001, Ley 2006, Caswell & Archambault 2008, Kleven 2008, Nicholas *et al.* 2008). These are generally not mutually exclusive and are used in combination as required.

Farming with *Mycoplasma* spp-free stock is economically advantageous as the cost of treatment and prevention is circumvented. It requires a strict biosecurity program however, effective surveillance program, knowledge of the epidemiology of the disease and, usually, *Mycoplasma* spp -free stock to start (Kleven 2008). At this stage too little is known about the epidemiology, particularly disease reservoirs and vertical transmission, to formulate an evidence-based eradication strategy for crocodile mycoplasmosis. Investigation of the epidemiological characteristics, requires, among other things, diagnostic tools to monitor pathogen exposure, host reaction to the pathogen, pathogen shedding by an infected host etc. PCR and serological assays are commonly used diagnostic tools, and the serological assays used in this trial are discussed in more detail in the last section.

Medication, including parenteral treatment of diseased crocodiles and/or in-feed treatment, have been performed during crocodile mycoplasmosis outbreaks, but treatment failures (Mohan *et al.* 2002), reports on antimicrobial resistance (Ayling *et al.* 2000, Reinhardt *et al.* 2002, Rosenbusch *et al.* 2005, Antunes *et al.* 2007) and high costs eliminates this as long term control strategy.

Vaccination against mycoplasmosis is widely used in commercial pig, poultry and cattle production systems, particularly in multi-age set-ups because it often is the only viable long-term option. Both inactivated and live-attenuated vaccines have been tested and are currently in use (Nicholas *et al.* 2009). The focus of the following discussion will be on inactivated vaccines as this is the type of vaccine dealt with in this study.

Vaccination

Vaccines to control animal mycoplasmosis had been in use even before the class *Mollicutes* was isolated or described (Nicholas *et al.* 2009). The first vaccination regimens against CBPP involved the insertion of infectious lung material subcutaneously in the base of the tail or the bridge of the nose of

cattle and, although the animal was reportedly protected against subsequent disease insults, resulted in severe adverse reactions such as loss of the tail or development of a horn-like bony outgrowth (Blancou 1996). This “vaccine” was neither inactivated nor attenuated and emphasizes the importance of vaccine safety.

Inactivated vaccines have been the first type of vaccine to be evaluated for most *Mycoplasma* infections because of the inherent safety thereof. Inactivated vaccines currently in use for major *Mycoplasma* spp infections include *M. hyopneumoniae* in swine, *M. gallisepticum* in poultry, *M. pneumoniae* in humans, *M. capricolum capripneumoniae* in goats and *M. agalactiae* in small ruminants (Nicholas *et al.* 2009). Most, if not all, of these are composed of inactivated, whole-cell adjuvanted vaccines, which are prescribed for either subcutaneous or intramuscular administration, at least twice before exposure to the pathogen, with periodic booster vaccinations suggested for some (Nicholas *et al.* 2009).

Despite the encouraging results obtained with inactivated vaccines, particularly concerning production parameters in poultry, swine and bovines (*M. gallisepticum*: Hildebrand *et al.* 1983, Glisson & Kleven 1984, Yoder & Hopkins 1984, Glisson & Kleven 1985, Yoder *et al.* 1985, Karaca & Lam 1987, Yogahashi *et al.* 1987, Barbour & Newman 1990, Elfaki *et al.* 1993, Nakamura *et al.* 1995) (*M. hyopneumoniae*: Maes *et al.* 1998, Maes *et al.* 1999, Thacker *et al.* 2000, Dawson *et al.* 2002, Siugzdaite & Garlaite 2002, Baccaro *et al.* 2006, Siugdiate *et al.* 2006, Maes *et al.* 2008) (*M. bovis*: Chima *et al.* 1980, Howard *et al.* 1987, Nicholas *et al.* 2002, Cho *et al.* 2008, Maunsell *et al.* 2009, Soehnlan *et al.* 2011), these vaccines fail to prevent host colonization/infection, the establishment of a carrier state, the spread of the organism, or cure of previously infected animals (Yoder & Hopkins 1984, Kleven 1985, Talkington & Kleven 1985, Yoder *et al.* 1985, Khan *et al.* 1986, Maes *et al.* 1998, Thacker *et al.* 1998, Ley 2006, Meyns *et al.* 2006, Kleven 2008, Villareal *et al.* 2011). It has been suggested that the systemic immunity that is induced is effective in minimizing the systemic effects of host infection, which influences parameters such as average daily gain, feed conversion ratio, egg production etc. (Razin 2006).

Fewer studies have been performed on the efficacy of inactivated vaccines against arthritic mycoplasmosis, but in general, favourable responses to vaccination have been reported (Chima *et al.* 1980, Washburn & Weaver 1997, Nicholas *et al.* 2002)

Unfortunately, disappointing results have been reported with inactivated vaccines against various mycoplasmas, the best described being *M. pneumoniae* in humans (Linchevski *et al.* 2009). Strain variation and *in vivo* antigen variation (discussed above) are two of the inherent characteristics of *Mycoplasma* spp that could complicate the use of inactivated vaccines. Furthermore, there have been reports suggesting that host immunity may exacerbate pathology (Poumarat *et al.* 1999 cited by Nicholas *et al.* 2008b, Bryson *et al.* 2002).

In summary, inactivated vaccines have been successfully used in the control of the negative effects of mycoplasmosis in some species, but various constraints have been reported. It is therefore difficult to predict or extrapolate the efficacy of an inactivated vaccine to a novel host and parasite.

Serodiagnosis of mycoplasmosis

Serological assays are often used to test animals for exposure to infectious agents, and include many of the common laboratory procedures such as the enzyme-linked immunosorbent assay (ELISA), agglutination, precipitation, neutralisation etc. Serological assays are preferred as they are often less time consuming and costly, and can be performed on live animals. Serology is also used to study disease epidemiology (Dawo & Mohan 2007) and to evaluate the efficacy of vaccination, particularly if protective antibody titres have been determined. Very few serological assays have been developed for infectious diseases of reptiles. A major constraint for these tests is the requirement for reptile-specific diagnostic reagents, which are not commercially available (Jacobson & Origi 2002).

For the diagnosis of crocodile mycoplasmosis, two serological assays, indirect ELISA and immunoblotting, have been developed (Dawo & Mohan 2007, Dawo & Mohan 2008). Unfortunately, neither of these tests is commercially available and therefore a locally developed latex agglutination test, and a growth inhibition assay were used in this trial.

Latex agglutination test

The latex agglutination test (LAT) is based on the observation of visible clumps, which form when cross-linking of antigen (attached to latex beads) by antibody (in test serum) results in the formation of visible aggregates (Gella *et al.* 1991, Stanley 2002). The use of coloured latex particles facilitate the observation of aggregates (Stanley 2002).

LAT is used as a screening test as it is simple, inexpensive, fast to perform, does not require sophisticated equipment and can, therefore, be used as a pen-side test (Rurangirwa *et al.* 1987, Gella *et al.* 1991, Nicholas *et al.* 2000, Gasparyan 2002, Stanley 2002).

Unfortunately, LAT has several weaknesses, including inconsistencies in endpoint readouts, cross-reaction with other antigens and variations in test performance due to batch variation (Gella *et al.* 1991, Stanley 2002). It has also been found that the main antibody detected by agglutination is IgM (Karpelin *et al.* 1993, Rastawicki *et al.* 2002, Kleven 2006), as this pentameric antibody is more effective in cross-linking several particles, thus forming larger clumps, which are more readily identified (Stanley 2002).

Agglutination tests have been described for many *Mycoplasma* spp infections and, despite the acknowledged constraints, are currently used in determining exposure to *M. gallisepticum* and *M. synoviae* (rapid serum plate agglutination assay), and *M. capricolum* subsp. *capripneumoniae* (latex agglutination) (OIE 2008a, OIE 2008b).

Metabolic inhibition assay

Growth inhibition (GI) assay is the preferred serological technique for the characterization of a new Mollicute species, and the metabolic inhibition (MI) assay is a modification of this assay (Whitcom *et al.* 1995, Brown *et al.* 2007). GI assay is based on the principle that antibody specific to the *Mycoplasma* species will inhibit the *in vitro* growth thereof (Taylor-Robinson *et al.* 1965). The MI assay, on the other hand, make use of the principle that certain *Mycoplasma* species metabolize glucose (resulting in lowering of the pH of the medium), and evaluate the metabolism (and consequently the *Mycoplasma* growth) by including a pH indicator in the growth medium (Taylor-Robinson *et al.* 1965). Although the GI assay is the generally recommended assay and the MI assay is suggested as alternative only for species that do not grow easily (Whitcom *et al.* 1995, Brown *et al.* 2007), it has been found that there is a close relation between growth inhibiting antibody and metabolism inhibiting antibody (Purcell *et al.* 1967)

Growth inhibition assays are highly specific for the *Mycoplasma* species and used to differentiate species (Black 1973, Whitcom *et al.* 1995, Brown *et al.* 2007). Unfortunately, these assays are laborious and difficult to perform, and media contamination could obscure results.

CHAPTER 3: EXPERIMENTAL DESIGN AND METHODS

Facilities and experimental animals

Study subjects

A group of approximately four thousand four hundred yearling farmed Nile crocodiles (*Crocodylus niloticus*) (22-24 months-of-age) was identified as the study subjects. The crocodiles were all part of the rearing stock of the Crocodile Unit of Nuanetsi Ranch, Mwenezi, Zimbabwe. These animals were bred in captivity at Nuanetsi Ranch.

The yearling crocodiles used in this study were all housed in House 6 (See farm layout and housing conditions below) during the period of vaccination. Of this group, two thousand two hundred crocodiles (housed in eight of the thirty yearling pens in house 6) were vaccinated with the experimental vaccine and were regarded as the experimental group. The remainder of the yearling crocodiles in this specific house was left unvaccinated and served as the control group.

All study subjects were moved into grower pens (See farm layout and housing conditions below) during June 2011. Each of the two pens in the grower houses were stocked with approximately one thousand one hundred vaccinated and one thousand one hundred unvaccinated (control) crocodiles.

Housing conditions

House 6 is one of four yearling houses. These are all divided into thirty smaller pens, arranged in six rows of five pens each, with walkways between rows 1 and 2, 3 and 4, 5 and 6 (fig 3.2). Each pen contains between 200 and 250 crocodiles, at a stocking density of 9 crocodiles per square metre. Each pen has two water ponds, each approximately 30 cm deep; approximately 50% of the floor area of the pen is occupied by the water ponds. Feed is provided on the concrete between the two water ponds. The entire pen is lined by concrete and pens are separated by a 50cm-high concrete wall. Shade cloth is used to cover ponds and these are opened and closed based on weather conditions.

The grower houses used for this experiment (pens 9B and 11B) are two of the grower crocodile pens in the unit. Each grower house consists of two pens (A and B), each with the capacity to house two thousand one hundred to two thousand two hundred grower crocodiles at a stocking density of 1 crocodile per square metre. Each pen has three water ponds, each approximately 50 cm deep and approximately 50% of the pen floor area is occupied by water ponds. Similar to the yearling pens, grower pens are also lined with concrete and pens are separated by a 100-120cm high concrete wall, but no shade is provided in these pens.

Nutrition, Feeding and watering

Feeding of yearling and grower crocodiles is done once daily. The diet is primarily meat based, but trace minerals, limiting amino-acids, carbohydrates and lipids are also included. The amount fed per day is determined by the amount of feed consumed. Breeding stock is fed once a week on a meat-based diet.

No formal quality control inspection is performed on feed ingredients on arrival.

The water used on the farm is pumped from the nearby Runde River. There is no formal quality control performed on the water; and no chemical or physical water treatment is done on water before using it in the Crocodile Unit.

Daily care of animals

The daily care of the crocodiles was performed by the personnel of Nuanetsi Ranch. As this trial was performed to evaluate the efficacy of the vaccine under field conditions, the farm-personnel was asked to treat experimental animals exactly the same as all other crocodiles.

Handling and restraint of crocodiles

All handling and restraint of crocodiles were performed by the personnel of Nuanetsi Ranch. Crocodiles were manually restrained for application of vaccine during the safety testing (see below) and for the application of the primary vaccination.

Farm layout

A schematic representation of the Crocodile Unit of Nuanetsi is presented in figure 3.1. The entire farm is surrounded by a diamond mesh and barbed wire perimeter fence. The main gate is guarded at all times and controls access to the majority of the operation. The crocodile ponds are separated from the rest of the farm by an additional fence. A total of sixteen houses are present. Houses 1 to 4 house hatchling crocodiles, houses 5 to 8 house yearling crocodiles, and houses 9 to 16 are built for grower crocodiles. The abattoir and feed-production facility is located approximately 300m from the crocodile's houses.

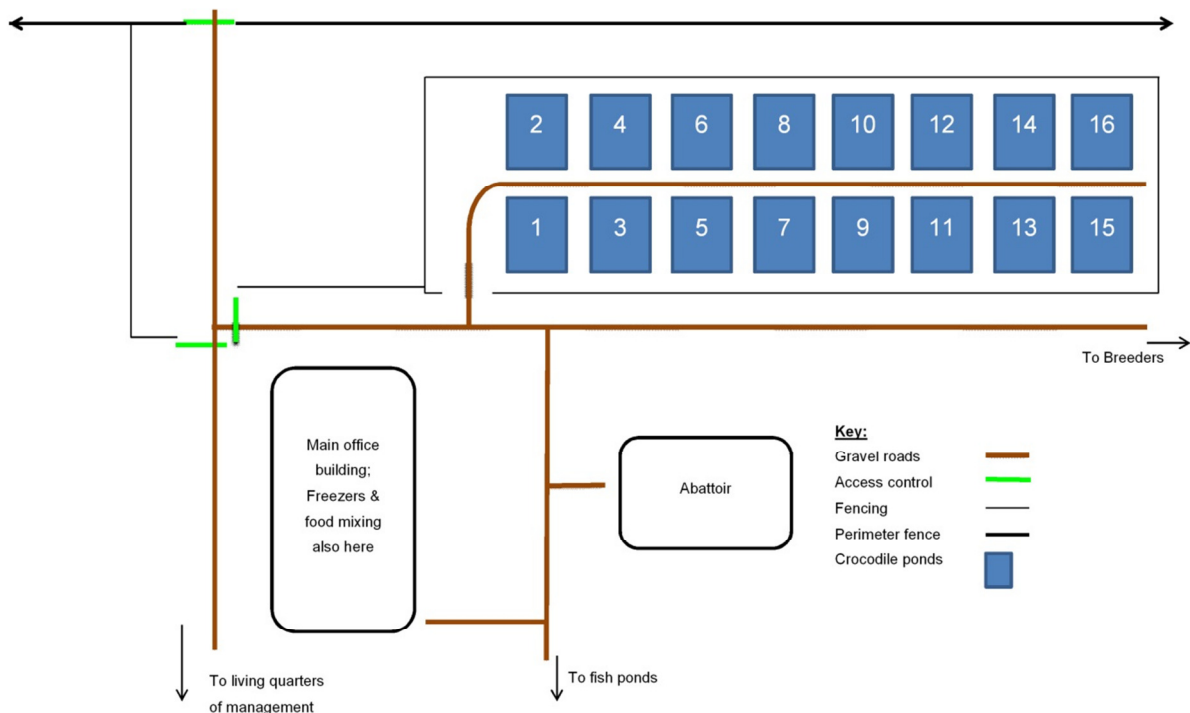


Fig. 3.1 **Layout of crocodile unit of Nuanetsi Ranch, Mwenezi, Zimbabwe**

Cleaning and disinfection

In the yearling crocodile pens, every pen is cleaned daily; this includes the removal of excess feed and faeces from the concrete surfaces and draining of the ponds.

In the grower crocodile pens, one of the three ponds is drained per day; the drained pond is then left empty for the day – the reasoning behind this being that the ultraviolet light from the sun will supply a sterilising effect on the pond. Practically this would equate into a single pond being empty for one day and filled for two days in every three-day cycle.

The farm employs an all-in-all-out system for an entire pen between batches of crocodiles being moved from the yearling to the grower pens, or from the hatchling to the yearling pens. After removal of all crocodiles, the pens are cleaned from all organic material, washed with Chlor-clean (Guest Medical Limited) and sprayed with Vircon® S (DuPont Chemical Solutions Enterprise). These chemicals are registered disinfectants. The active ingredients present in Chlor-clean is trocose sodium (decomposing to chlorine (Cl₂), hypochlorous acid (HClO) and cyanuric acid ((HOCN)₃ on contact with moisture) and in Vircon® S potassium peroxomonosulfate (KHSO₅ – an oxidising agent), sodium dodecylbenzene sulphonate (C₁₂H₂₅C₆H₄SO₃Na – a surfactant) and sulfamic acid (H₃NSO₃). The pen is left empty for a minimum of 10 days after cleaning, before new crocodiles are moved in. Due to circumstances, such as an increase in the numbers of crocodiles, it is not always practically to follow this cleaning regime. The two grower pens in which the study crocodiles were kept were however, subjected to this cleaning regime.

Biosecurity

Access control to the farm as well as to the crocodile pens is practised. Personnel and visitors are expected to step into a footbath before entering crocodile pens. Dead crocodiles are removed from pens on a daily basis. Pens are cleaned daily as described above. Separate cleaning equipment for different pens are supplied but not necessarily used. Feed transport crates are shared between pens. Natural vermin control by cats is practised. On a previous occasion yearling crocodiles had been bought from other crocodile farms and co-mingled with Nuanetsi crocodiles without practicing quarantine before introduction; this was followed by the first outbreak of suspected mycoplasmosis.

Vaccine production

Mycoplasma-strain included in experimental vaccine

The isolate used for the preparation of the vaccine was cultured from the joint fluid of a sick crocodile (crocodile no. 2), which was euthanized during a visit to Nuanetsi Ranch during August 2010. It was identified as *M. crocodyli* by means of growth inhibition of mono-specific antisera and an indirect fluorescent antibody test.

Vaccine production and formulation

The isolate was cultured in a modified Hayflick's broth (Hayflick 1965) and inactivated with 0.4% formalin for 3 days at 37°C. The antigen titre was adjusted to a minimum of 10^8 cfu/ml. Aluminium hydroxide (at a concentration of 33%) was added as adjuvant. The final product was bottled in 100ml vaccine vials and supplied ready-for-use to the farmer.

Quality control of vaccine

After formulation, the vaccine was tested for sterility by DESIGN BIOLOGIX, Pretoria, South Africa.

Safety testing of the autogenous vaccine

To evaluate the safety of the autogenous vaccine, it was administered intraperitoneally to a subset of twenty crocodiles one month before commencement of the vaccine immunogenicity phase. Of these crocodiles, five were given a single dose of 2 ml of the vaccine, five received a double dose, and ten were sham vaccinated controls and received 2ml sterile water intraperitoneally. The crocodiles were permanently marked by removal of the scutes from the left side of the tail; for the single vaccine dose a single scute was removed from the left side, for the double vaccine dose two scutes were removed from the left side and for the control animals three scutes were removed from the left side.

Crocodiles were evaluated daily for signs of disease, mainly lethargy and anorexia, and recorded by personnel from Nuanetsi Ranch.

Efficacy testing of the autogenous vaccine

To evaluate the efficacy of the experimental vaccine, two thousand two hundred yearling crocodiles were vaccinated with the experimental vaccine. The other two thousand two hundred yearling crocodiles were kept as unvaccinated control animals. A schematic representation of the trial is provided in Figure 3.2 below.

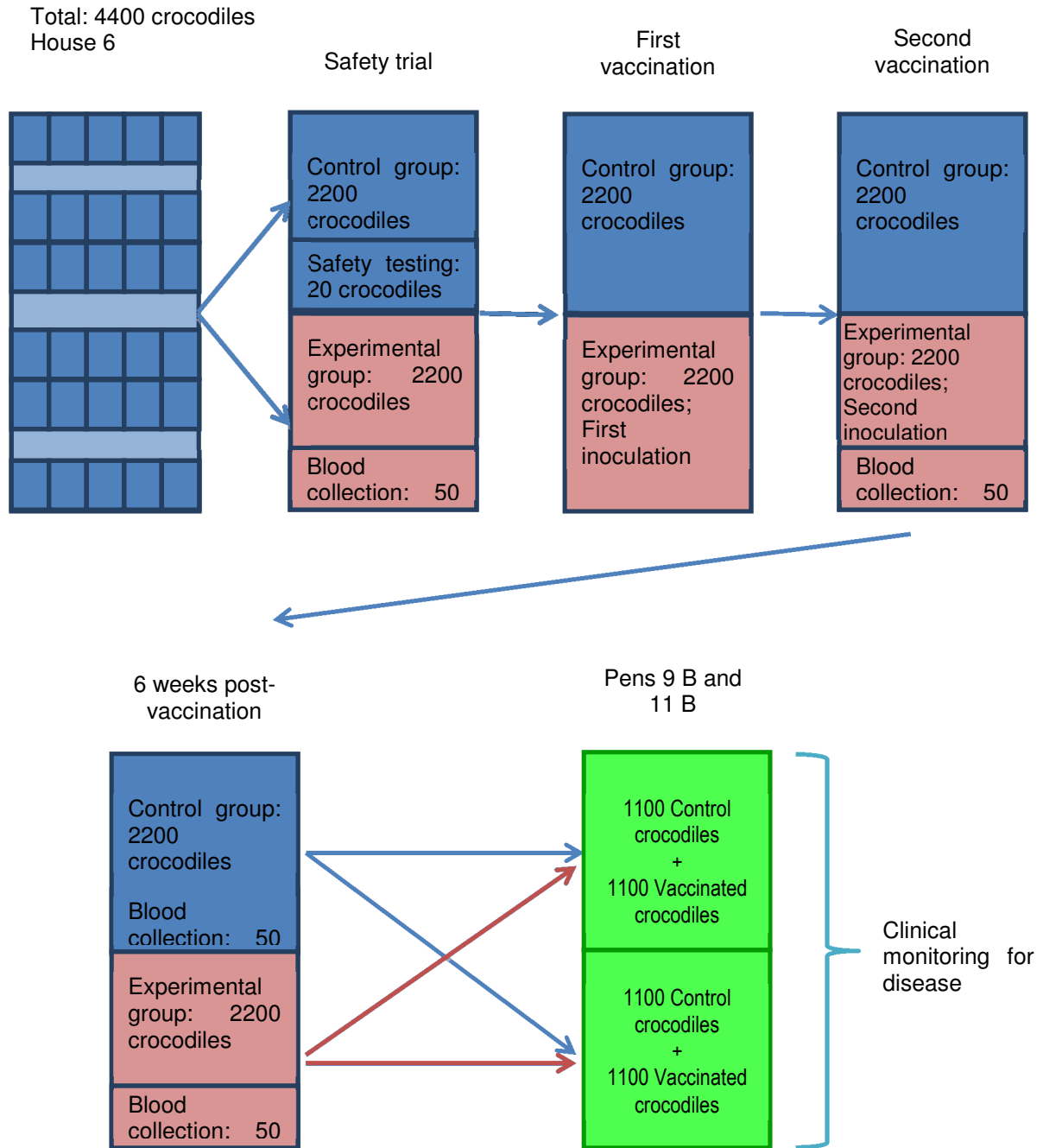


Fig. 3.2: Schematic representation of trial

The experimental vaccine was administered intramuscularly in the deep muscles of the tail (either the caudal femoral muscle (ventral to the transverse process of the vertebrae) or the *m longissimus caudalis* (dorsal to the transverse process of the vertebrae) (Richardson *et al.* 2002, Huchzermeyer 2003). A dose of 1 ml vaccine per animal was administered with a 20G needle. The needle was changed after every ten animals, or sooner if it became damaged.



Fig. 3.3 Vaccination of crocodiles. The vaccine was administered intramuscularly in the tail.

All vaccinated crocodiles were permanently marked by removal of one scute from the right side of the row of double scutes on the tail at each vaccination. See figure 3.4 below for clarification.



Fig. 3.4 Crocodile tail indicating the healed lesion after removal of one scute from the right.

The post mortem examination was done according to the procedures described by Huchzermeyer (2003). Particular attention was given to the joints of the appendicular skeleton.

Serum collection for serological testing

Sampling points for serum collection

Five points for serum collection were identified. These were: (1) prior to administration of the first vaccination, (2) four weeks after first vaccination, at the time of second vaccination, (3) at least four weeks after the second vaccination, (4) approximately six months after the second vaccination, and

(5) during and/or after an outbreak of disease. Samples from the experimental group were taken at all five points, while samples from the control group were only taken at points 3, 4 and 5. Approximately fifty samples were collected from each group at sampling points 1, 2 and 3.

Sampling points 4 and 5 represents sampling during a disease outbreak. Samples at sample point 4 were collected from approximately 50 vaccinated animals with clinical disease. Samples at sample point 5 were collected from approximately 50 vaccinated animals without clinical disease, 50 unvaccinated animals with clinical disease and 50 unvaccinated animals without clinical disease.

Procedure for serum collection

All samples were collected from electrically stunned crocodiles except for the samples collected pre-vaccination which were collected from crocodiles restrained manually.

The samples were collected from the dorsal coccygeal vein, as previously described by Huchzermeyer (2003). In short, the procedure entails identification of the correct area, careful insertion of the hypodermic needle through the intervertebral ligament, and slow application of suction to the syringe to draw the blood into the syringe. The sample size ranged from 5 to 10 ml of blood. Samples were collected with a 20G to 21G 1.5" hypodermic needle, and a 5 or 10ml single-use syringe. Each animal was sampled with a separate needle and syringe. The collected blood was transferred to a serum tube (SG-vac), where the blood was allowed to clot.



Fig. 3.5 Collecting blood from a crocodile.

Procedure for serum processing and transport to laboratories

After removal of the serum from the blood clot by the personnel from the Wildlife Veterinary Unit laboratory, Harare, Zimbabwe, the samples were stored at -18°C until transport to the DSTD bacteriology laboratory.

Latex slide agglutination test

Latex slide agglutination was performed as a screening test to detect sero-positive samples. Serial two-fold dilutions were made of all sero-positive samples to determine the titre of the samples. The reciprocal of the last dilution where agglutination could be observed, was taken as the titre.

Mycoplasma strains and growth conditions

The isolate (seed material) used for the preparation of the latex agglutination assay, was the same strain used to prepare the experimental vaccine

A culture of the organism was prepared in modified Hayflick's broth (Hayflick 1965) at 37°C. The culture was transferred every second day to fresh broth, with inocula of approximately 10%, at least three times, in order to prepare a final volume of approximately 4 litres.

Coating of Microspheres

A similar procedure to that published by Senthilkumar *et al.* (2008) for canine leptospirosis was used with slight modifications. The procedure is summarized below.

To prepare the culture for adsorption to the latex microspheres, the organism was inactivated by the addition of 0.4% formalin and incubation at 37°C for five days. Cells were concentrated by high speed centrifugation, at 15000rpm at 4°C for thirty minutes, washed twice in phosphate buffered saline (PBS pH 7.2) and re-suspended as 10ml per 1 litre *Mycoplasma* culture. The suspension was dialysed against PBS pH 7.2 for 24 hours at 4°C, and re-suspended to 100ml per 1 litre *Mycoplasma* culture, in PBS to which 0.1% sodium azide had been added.

A 10% suspension of latex beads (0.80 µm, SIGMA-ALDRICH) was added to the suspended *Mycoplasma* culture at a ratio of 1 part latex beads to 9 parts *Mycoplasma* culture. The suspension was gently stirred for 6 hours at 37°C, after which it was centrifuged at 8000 rpm at 4°C for three minutes. The resultant pellet was re-suspended in PBS pH 7.2 with 0.1% sodium azide, at a dilution of 10ml PBS pH 7.2 per 10ml latex-antigen suspension. The coated latex beads were stored at 4°C until use.

Procedure for latex slide agglutination

The methodology was adapted from Senthilkumar *et al.* (2008), and is similar to the LATs published for other *Mycoplasma* spp. (Morton 1966, Slavik & Switzer 1979, March *et al.* 2000, March *et al.* 2003). In short, the LAT was performed by pipetting 20µl of serum and 20µl of antigen onto a glass slide (approximately 22 samples were tested per run on a 150 x 210 mm glass slide, and positive and negative controls were included on each run)(all reagents at room temperature). The reagents were gently mixed and hand-rocked for approximately 2-3 minutes at room temperature, after which the test was read. If fine granular clumps formed in the mixture, a positive reaction was recorded, and if the suspension remained homogeneously pale blue, a negative reaction was recorded. For all positive

sera, the procedure was repeated with serial two fold serum dilutions to determine antibody titre. The titre of the serum was recorded as the reciprocal of the last dilution where a positive reaction could be distinguished.

Growth/metabolism inhibition assay

The metabolism inhibition assay (MI) was performed (a) to determine the titre of the sera which tested positive on LAT and (b) as a gold standard assay to evaluate the performance of the latex agglutination assay. The method employed was similar to that originally described by Taylor-Robinson *et al.* (1966), with some modification, and is briefly described below.

Mycoplasma strain and culture

For the MI test, the same strain as for the latex agglutination test which is described above was used. It was grown in modified Hayflick's medium (Hayflick 1965) at 37°C, and the culture was transferred to fresh broth every second day, with inocula of approximately 10% each time. The final transferral was performed just before performance of the MI test.

Mycoplasma medium

The organism was grown in modified Hayflick's medium (Hayflick 1965) with the addition of phenol red (phenolsulfonephthalein) (as pH indicator).

Procedure for MI test

The tests were performed on disposable, plastic 96-well microplates (NUNC 96F Untreated straight with lid, Thermo Fisher Scientific). All procedures were performed in a laminar flow cabinet to minimize contamination. Serial two-fold serum dilutions were prepared with *Mycoplasma* medium, to a volume of 100µl. 100µl of the prepared *Mycoplasma* culture was then added to each well, to obtain a final serum two-fold dilution series from 1:2 to 1:256. The microplates were sealed with adhesive, see-through plastic and incubated at 37°C for 48-54 hours. The reactions were read when obvious colour changes (pink to yellow) could be observed. A positive result was read when the colour remained pink (i.e. metabolism of glucose was inhibited and the pH remained constant) while a negative result was read if the colour changed from pink to yellow (i.e. metabolism of glucose and a decrease in pH occurred). The titre of the serum was read as the reciprocal of the last dilution where glucose metabolism was inhibited (i.e. no colour change could be observed). The remaining *Mycoplasma* broth was also incubated as control to determine the sterility of the broth.

Statistical evaluation of results

Data was captured in Microsoft Excel 2010 spreadsheets and analyses were carried out with the aid of MINITAB Statistical software (Release 13.32) and Microsoft Excel spreadsheets.

Evaluating sero-conversion

The strength of association between vaccination and serological status was evaluated. The proportions of sero-positive crocodiles before vaccination, 4 weeks after primary vaccination and 6 weeks after booster vaccination were compared using a Pearson's chi-square test.

A significance level of $\alpha = 0.05$ was used for all comparisons

Evaluating the association between serological status, disease status and vaccination status during disease outbreak

The strength of association between the ranked titre, disease status and vaccination status of crocodiles at the time of the outbreak was compared using stepwise regression. Association was assessed at a significance level of $\alpha = 0.05$.

Evaluating the diagnostic performance of the latex agglutination test

The diagnostic performance of the latex agglutination test had not been evaluated previously for a large sample size and it was therefore, calculated from the data generated in this study. As MI tests were not performed for all sera on which LAT was performed, only the subset of sera on which both tests had been performed for all 4 sampling points were used to calculate this data. The sensitivity, specificity, positive and negative predictive values were calculated according to standard formulae (Thrusfield 2005), and are provided below:

Table 3.1 2x2 contingency table for determining diagnostic performance

	Gold standard positive	Gold standard negative	Total
Test positive	a	b	a+b
Test negative	c	d	c+d
Total	a+c	b+d	a+b+c+d

- Sensitivity = $\frac{a}{a+c}$
- Specificity = $\frac{d}{b+d}$
- Positive predictive value = $\frac{a}{a+b}$
- Negative predictive value = $\frac{d}{c+d}$
- Accuracy = $\frac{a+d}{a+b+c+d}$

CHAPTER 4: RESULTS

Safety trial

None of the crocodiles in the group used to evaluate the safety of the vaccine displayed any signs of an adverse vaccine reaction, such as lameness, reduced appetite or death.

Efficacy trial

Latex agglutination assays

A latex agglutination assay (LAT) was performed on all samples. The layout of a typical plate is demonstrated in Figure 4.1. Examples for positive and negative results are given in Figure 4.2 and Figure 4.3 respectively. A Summary of the results is presented in Table 4.1 below.

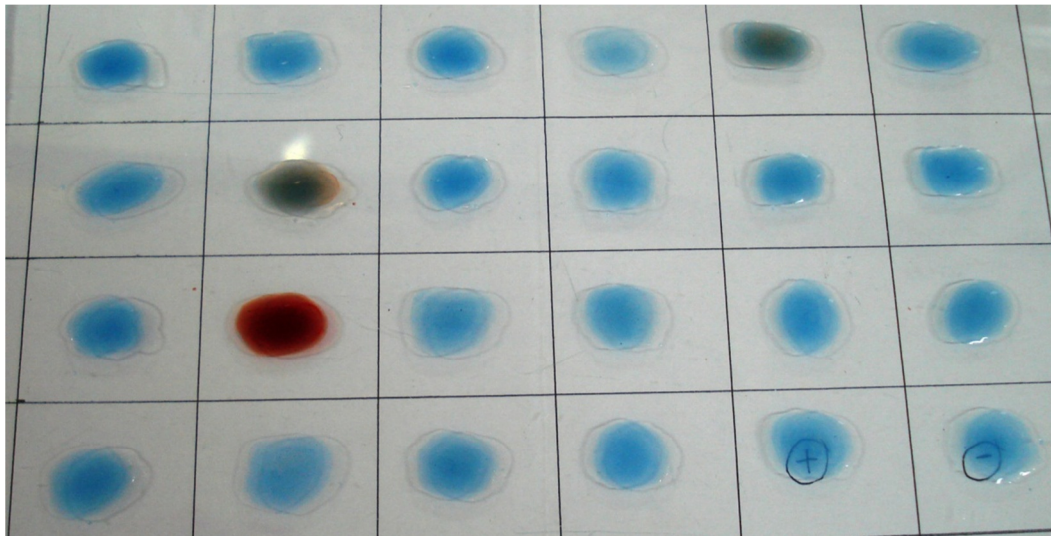


Fig 4.1 Layout of a typical LAT plate. In the right bottom corner the positive and negative controls can be seen.

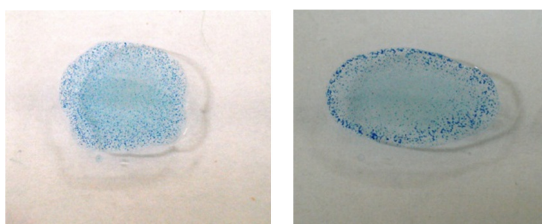


Fig. 4.2 Two examples of positive reactions

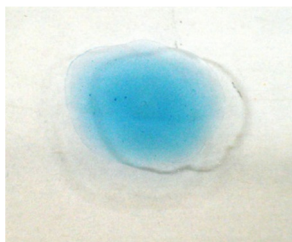


Fig. 4.3 An example of a negative reaction.

Table 4.1 **Summary of LAT results**

	Pre- vaccination	4 weeks post primary vaccination	6 weeks post booster vaccination	6 weeks post booster vaccination
Vaccination status of animals	N/A	Vaccinated	Vaccinated	Unvaccinated
Number of seropositive animals	4	24	12	9
Number of seronegative animals	67	26	38	40
Total number of animals	71	50	50	49
% Seropositive animals:	5.63%	48.00%	24.00%	20.41%
95% Confidence interval for seropositive animals	1.56- 13.80%	33.66 - 62.58%	13.06- 38.17%	8.76-32.02%

From this data it can be seen that:

- almost 6% of the tested animals were seropositive before the vaccine was administered;
- 48% of vaccinated animals were seropositive 4 weeks after the first vaccination;
- 24% of vaccinated animals were seropositive 6 weeks after the booster vaccination;
- 20% of unvaccinated animals were seropositive 6 weeks after the booster vaccination.

Growth/metabolic inhibition assays

A summary of the results of the metabolic inhibition assays are presented in Table 4.2 below.

 Table 4.2 **Summary of MI test results**

	Pre- vaccination	4 weeks post primary vaccination	6 weeks post booster vaccination	
Total number of animals	50	23	17	10
Vaccination status of animals	Unvaccinated	Vaccinated	Unvaccinated	Vaccinated
Number of seropositive animals	5	0	14	8
% Seropositive animals:	10.00%	0.00%	82.35%	80.00%

In summary this data shows that:

- 10% of crocodiles were seropositive with the MI test before vaccination, although the titres were low;
- None of the vaccinated crocodiles were seropositive with the MI test 4 weeks after the first vaccination;

Results from outbreak, October 2011

Pathology

Necropsies were performed on nine crocodiles during the outbreak of disease in October 2011. Four crocodiles had severe polyarthritis, particularly of the appendicular skeleton. The pathology ranged from acute, fibrino-serous arthritis with swelling and oedema of the surrounding tissue, to chronic, purulent arthritis with hyperplasia of the joint capsule (See Figure 4.4, 4.5 and 4.6). Skin abrasions on the feet and over bony prominences, were also present (See Figure 4.7).



Fig. 4.4 **Acute arthritis.** An intra-articular sero-fibrinous exudate is present, particularly in A. Swelling of the surrounding tissue can also be seen, particularly in B.



Fig. 4.5 **Subacute arthritis.** Intra-articular exudate changed to fibrino-purulent and fluid decreased.

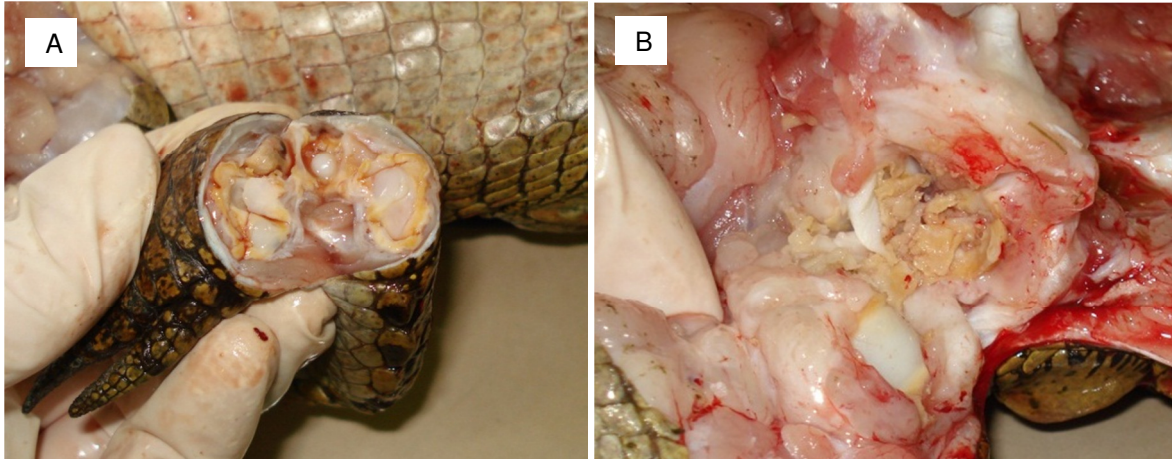


Fig. 4.6 Chronic arthritis. Purulent intra-articular exudate is present.

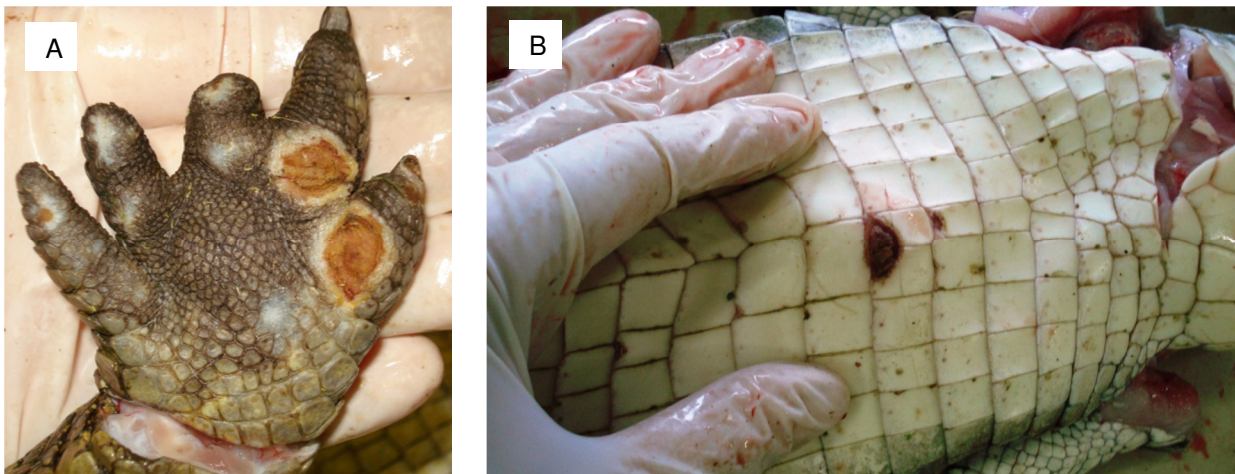


Fig. 4.7 Typical skin lesions on the feet (A) and over the sternum (B).

Serology

The results of LAT and MI assays on serum collected during the disease outbreak are summarized in Table 4.3 and 4.4 below.

Table 4.3 Summary of Outbreak results-LAT

Vaccination status of animals	Vaccinated	Unvaccinated	Vaccinated	Unvaccinated
Disease status of animals	Diseased	Diseased	Healthy	Healthy
Total number of animals	41	48	50	53
Number of seropositive animals	29	41	42	40
% Seropositive animals:	70.73%	85.42%	84.00%	75.47%
95% Confidence interval for seropositive animals	54.46-83.87%	72.24-93.93%	70.88-92.83%	61.72-86.24%
% Seronegative animals:	29.27%	14.58%	16.00%	24.53%

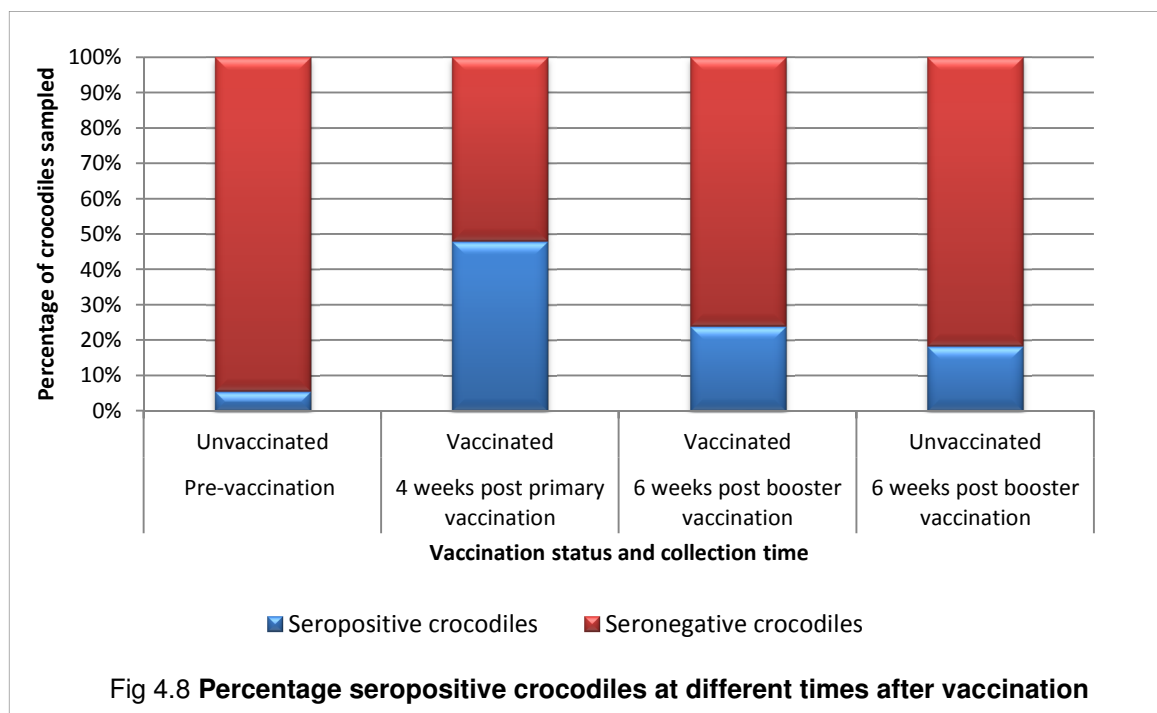
Table 4.4 Summary of Outbreak results-MI test

Vaccination status of animals	Vaccinated	Unvaccinated	Vaccinated	Unvaccinated
Disease status of animals	Diseased	Diseased	Healthy	Healthy
Total number of animals	37	47	48	48
Number of seropositive animals	21	10	23	15
% Seropositive animals:	56.76%	21.28%	47.92%	31.25%
95% Confidence interval for seropositive animals	39.49-72.90%	10.70-35.66%	33.29-62.81%	18.66-46.25%
% Seronegative animals:	43.24%	78.72%	52.08%	68.75%

Statistical evaluation of results

Evaluating the relationship between vaccination and seroconversion

A graphical presentation of serological status (determined by LAT) at the different time periods is provided in Fig. 4.8



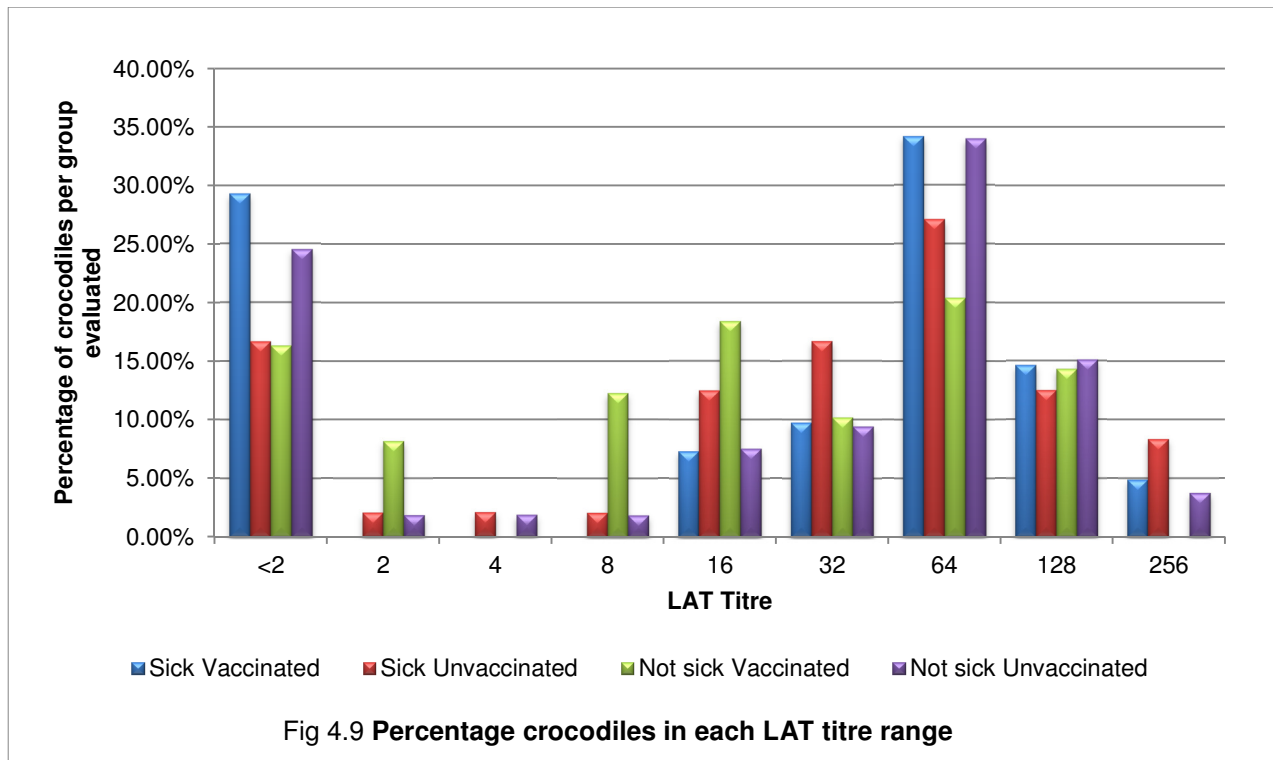
At the pre-vaccination 5.63% of (unvaccinated) crocodiles (95% Confidence interval (CI) = 1.16% to 13.80%) were seropositive while 48.00% vaccinated crocodiles (95% CI = 33.66% to 62.58%) were seropositive 4 weeks after the primary vaccination. This represents a statistically significant increase in seropositive crocodiles ($p < 0.001$).

Six weeks after booster vaccination 24.00% vaccinated crocodiles (95% CI = 13.06% to 38.17%) and 18.37% unvaccinated crocodiles (95% CI = 8.76% to 32.02%) were seropositive. Compared to the pre-vaccination serological status, this represents a statistically significant increase in seropositive

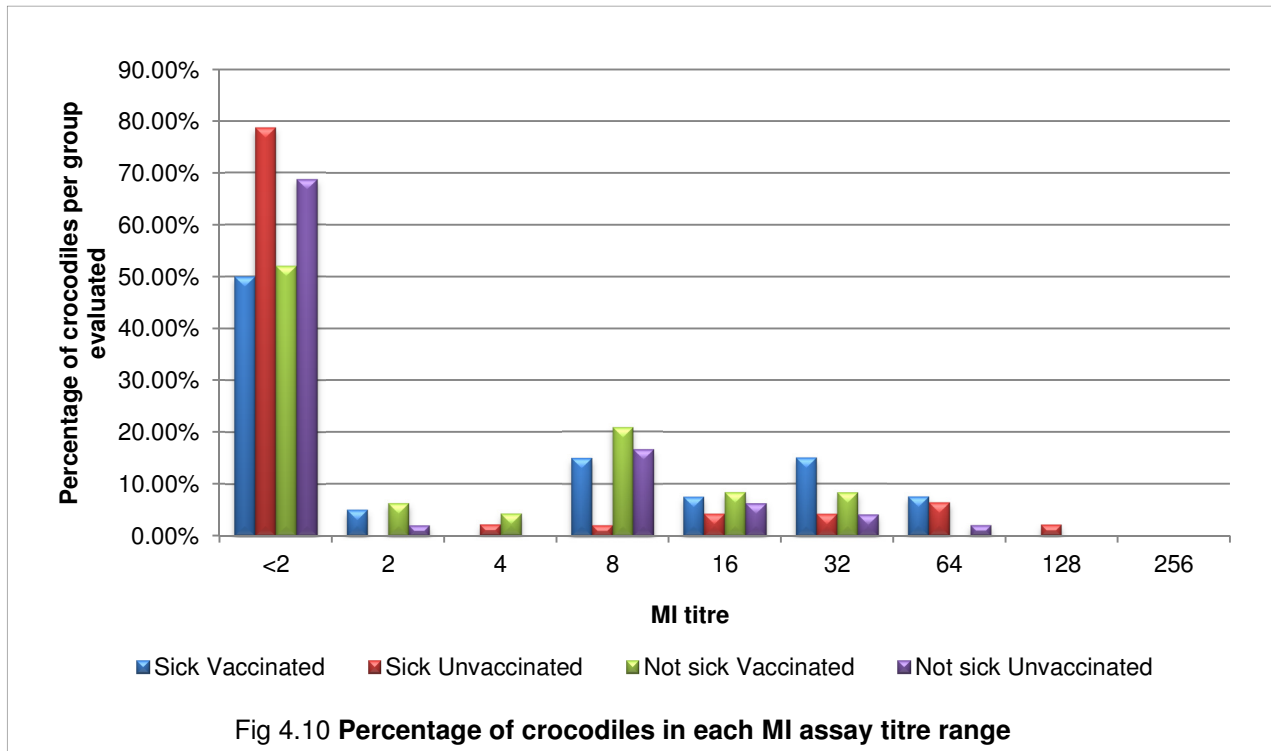
crocodiles for both groups ($p < 0.05$). However, when comparing the vaccinated and unvaccinated groups with each other, there is no statistically significant difference in seropositive animals ($p > 0.05$). Furthermore, when comparing the proportion of seropositive vaccinated crocodiles 6 weeks after the booster vaccination there is a statistically significant decrease compared to the proportion of seropositive vaccinated crocodiles 4 weeks after the primary vaccination ($p < 0.05$).

Evaluating the relationship between vaccination status, serological status and development of clinical disease during a disease outbreak

A graphical presentation of the percentage of crocodiles represented in each serum dilution tested with the LAT and MI assay is presented in figures 4.9 and 4.10 respectively.



From the graph in Fig. 4.9 it can be said that there is no clear pattern in the titre of sampled crocodile groups, regardless of the vaccination and/or disease state. This was confirmed by performing a stepwise regression to assess the dependence of the ranked LAT titre to disease status, vaccination status and/or the interaction between these variables ($p > 0.05$, $r^2 < 5$).



Calculation of the dependence of the ranked MI titre on disease status, vaccination status and the interaction of these variables, indicated that, similar to the LAT assay, the serological titre was independent of these variables ($p > 0.05$, $r^2 < 5$).

Evaluating the diagnostic performance of the latex agglutination test

The diagnostic performance of the latex agglutination test was determined by using the MI test as the gold standard.

Table 4.5 Relationship between LAT results and MI test results

	MI test positive	MI test negative	Total
LAT positive	68 (a)	122 (b)	190 (a+b)
LAT negative	28 (c)	62 (d)	90 (c+d)
Total	95 (a+c)	185 (b+d)	280

From this data the following parameters were calculated according to the formulas provide in Chapter 3:

- Diagnostic sensitivity = 72%
- Diagnostic specificity = 32 %
- Predictive value of the positive test = 36%
- Predictive value of the negative test = 69%
- Overall test accuracy = 46%

CHAPTER 5: DISCUSSION

The aim of this study was to determine whether protective immunity could be stimulated in farmed Nile crocodiles by the administration of an inactivated *Mycoplasma crocodyli* vaccine. The *in vivo* safety and immunogenicity as well as the efficacy in the face of a natural outbreak were evaluated.

Vaccine safety

The clinical data indicated that the vaccine did not cause any systemic adverse reactions and no increase in mortalities was reported for crocodiles in the study group. It is therefore concluded that the vaccine was safe for use in crocodiles.

To evaluate the safety of the vaccine, it was decided to inoculate the crocodiles intraperitoneally rather than intra-muscularly. The reasons for this are as follows: Firstly, the serosal surface of the peritoneum should be more sensitive to the presence of an irritant substance, and therefore more likely to result in notable adverse vaccine reactions, than the muscle. Secondly, if any live infectious *Mycoplasma* organisms were present in the vaccine, clinical disease was more likely to result when given intraperitoneally, as this was the most successful method of reproducing clinical mycoplasmosis (Mohan *et al.*1995).

Pathological and histopathological evaluation of the injection sites were not performed because the study subjects were part of the stock of a commercial crocodile farm and not available for sacrifice. When evaluating the safety of such a vaccine for commercial production, safety assessment by pathological and histopathological examination of the injection site as well as pathological examination of animals to evaluate systemic effect of vaccination, is recommended (EMEA 2001).

Vaccine efficacy

Immunogenicity

The first step in evaluating the efficacy of the vaccine was to determine if the vaccine could induce a humoral immune response. Several conclusions can be drawn from the serological data, presented in tables 4.1 and 4.2. Firstly, both tests (LAT and MI test) detected a small percentage of crocodiles sero-positive before the onset of vaccination, although no individual animal was positive on both assays. Secondly, there was a significant increase in the proportion of LAT seropositive crocodiles four weeks after primary vaccination but none of the crocodiles that were positive on LAT, at this time point, were also positive with the MI test. Furthermore, there was also an increase in LAT seropositive crocodiles when comparing pre-vaccination samples to samples collected six weeks after the booster vaccination. However, there was actually a significant decrease in the proportion of LAT seropositive crocodiles since the previous collection (four weeks after primary vaccination). In contrast, the proportion of LAT seropositive crocodiles that also tested positive with the MI assay increased after booster vaccination compared to either of the previous samplings.

Unfortunately, interpreting these results is obscured by the fact that only the pre-vaccination samples were evaluated with both assays. As set out by the study protocol, only LAT positive sera were meant to be evaluated with the MI test, which was the procedure followed for the samples collected after primary and booster vaccinations. A second serious constraint was that, although vaccinated crocodiles were explicitly marked and distinguishable from unvaccinated crocodiles, individuals could not be discerned, resulting in the inability to trace the serological and/or clinical response of individual crocodiles. Despite these constraints, it was felt that some hypotheses for the mentioned trends can be formulated, and these are discussed below.

The presence of seropositive samples before onset of the vaccination trial and in unvaccinated crocodiles during the trial is of interest. There are a few possible explanations for this finding. Firstly, it could indicate that the organism was present and circulating through the population during the study, and, therefore, that the organism is persistently present on the farm. This epidemiological situation seems feasible if one considers the information available for poultry and pig mycoplasmosis on multi-age production units (Desrosier 2001, Nicholas 2004, Ley 2006). If this were the case, our knowledge of the management of mycoplasmosis in the mentioned species would indicate that the use of an inactivated vaccine in such a situation could be beneficial to reduce losses but is unlikely to eliminate the problems (Desrosiers 2001, Ley 2006).

A second probable explanation for positive pre-vaccination samples is non-specific cross-reaction of serological assays. Non-specific reactions are well-described for the rapid serum plate agglutination assay used for *M. gallisepticum* (Ahmad *et al.* 1988, Avakian & Kleven 1990, Ross *et al.* 1990, Ben Abdelmoumen & Roy 1995). It would be ideal to evaluate the analytical specificity of a newly developed assay to quantify the occurrence of false positives due to cross-reactions. However, because a wide variety of, seemingly, unrelated antigens could be involved, as clearly demonstrated for the *M. gallisepticum* assay, determining analytical specificity is often only a theoretical concept. Running serological assays in series, is one of the methods employed to improve the specificity of a test.

The role of natural antibodies in pre-vaccination seropositive assays also requires clarification. As mentioned in chapter 2, it has been reported that natural antibodies to *Mycoplasmas* occur in tortoises and that these could play a complicating role in tortoise *Mycoplasma* serology (Hunter *et al.* 2008, Sandmeier *et al.* 2009). A similar situation in crocodiles should be considered, particularly because this could result in the erroneous diagnosis of a *Mycoplasma*-infected animal/group/farm, while the specific individual could simply have detectable “innate” immunity to *Mycoplasma*-like antigens.

These uncertainties make interpretation of the rest of the data-trends even more complicated. It was decided that, in order to draw conclusions, regard all seropositive cases will be viewed as having a true immune response to the administered antigen.

The increase and subsequent decrease in LAT seropositive crocodiles requires further consideration. One of the possible reasons for this observation is that the predominant antibody type detected by agglutination assays is IgM (Zimmermann & Ross 1982, Karppelin *et al.* 1993, Rastawicki *et al.* 2002, Kleven 2006). From an immunological point of view, it would make sense that a larger proportion of animals would have circulating IgM four weeks after the primary vaccination than 6 weeks after the booster vaccination (i.e. ten weeks after the primary vaccination). However, if one considers that Zimmerman *et al.* (2010) reported that reptilian IgM persists for more than 20 weeks after exposure, this statement does not hold true. Class switching, for which the details have not been studied in crocodilians, could be one explanation for this discrepancy in LAT results, if the predominant antibody subtype changed from IgM to IgY during the interim period. The predominant antibody type involved in MI against *M. crocodyli* is unknown but it has been reported that both IgM and IgG were involved in MI for *M. hyosynoviae* (Zimmermann & Ross 1982). Thus, a satisfactory, scientific explanation for the results obtained cannot be formulated from what is currently known about the reptile immune response, particularly to *Mycoplasma*.

Vaccine efficacy during a disease outbreak

DISEASE CHALLENGE

In light of the suspected endemic disease situation on the farm, evident in repeated outbreaks over the past few years, it was decided that vaccine efficacy would be assessed in the face of natural disease challenge, and artificial disease challenge (by active infection of crocodiles with virulent organisms) were therefore not originally planned. This approach is also suggested in the applicable EMEA guideline document for field trials of veterinary vaccines (EMEA 2001).

In contrast to expectations, no general outbreak of mycoplasmosis was reported for the winter of 2011, although sporadic cases were reported in August 2011. The first major outbreak was only encountered during spring and early summer, approximately 5 months after the booster vaccination. Because this outbreak only affected one pen of crocodiles on the entire farm (which were the same pen that had reported sporadic cases in August 2011) but did not house study animals, it was decided to introduce 50 sick crocodiles from the affected pen to each of the pens housing study crocodiles (approximately 4400 crocodiles per pen). Approximately 2 weeks after the introduction of the diseased crocodiles, clinical lameness and paralysis were also reported in study crocodiles. Although diseased animals had not been introduced to other houses, clinically diseased grower crocodiles were also detected in most of the other grower houses at this stage.

Before considering the outcome, a few aspects regarding the challenge need to be evaluated. Firstly, as for almost any field trial (EMEA 2001), the severity of challenge to which the crocodiles were exposed, was uncontrolled and is largely unknown. It is also not known if any other disease conditions were circulating in the population at the time of the outbreak. Nonetheless, the clinical disease and mortalities observed correlated with what was previously reported on this farm.

Secondly, the number of diseased crocodiles introduced into each pen was chosen arbitrarily and was mainly based on what was practical to perform. The main motivation behind this was the lack of information to make use of a more sophisticated method, as very little is known about the epidemiology of crocodile mycoplasmosis and the basic reproductive number (R_0) is therefore unknown.

A third complication related to disease introduction and study design, was the co-mingling of vaccinated and unvaccinated crocodiles. It has been stated that in a population consisting of both vaccinated and unvaccinated animals, the unvaccinated group amplifies the pathogen until an infectious pathogen load is reached which overwhelms the pre-induced immunity in the vaccinated group (Dohoo & Montgomery 1996, Maunsell *et al.* 2009). This was identified as a potential problem before the onset of the trial and it was suggested that close monitoring of the study crocodiles is performed in order to determine if a difference in the susceptibility to disease and the outcome of disease related to vaccination could be discerned. Unfortunately, clinical monitoring of study animals turned out to be more difficult than anticipated (see below).

Furthermore, the timing of this outbreak is of significance. In contrast to outbreaks in previous years, which usually occurred during cooler winter months (May to July/August), this outbreak occurred during hot weather. It was also not linked to the movement of crocodiles from yearling houses to grower houses, which was identified as a possible stressful event precipitating previous outbreaks of mycoplasmosis. The reason/s for these differences is unknown. Despite the uncertainties in the serological data (described above), it could theoretically be possible that vaccination stimulated partial, though short-term immunity and protected the group of grower crocodiles for a limited period. However, considering the complete lack of disease in the entire population, it seems more likely that disease challenge was either absent or that the correct combination of predisposing factors were absent.

Finally, the described time from introduction of infected crocodiles to disease in susceptible crocodiles correlates well with the described incubation time for swine and poultry mycoplasmosis (Desrosiers 2001, Ley 2006).

CLINICAL AND PATHOLOGICAL MONITORING

Because the study was performed on a Zimbabwean crocodile farm where the disease is endemic, it was not possible for the principal investigator to be present on the farm for the duration of the outbreak. Hence clinical observation, including the number of diseased animals, number of deaths, response to treatment and environmental monitoring, were left to the ranch manager. Due to the size of the operation and the magnitude of managerial tasks, the manager was unfortunately unable to complete the predetermined records.

During the outbreak, all clinically diseased crocodiles (according to the ranch managers' and workers' discretion, which usually entailed complete paralysis) from the entire operation were moved to the originally infected pen, in order to control the disease challenge to the rest of the operation. Unfortunately this resulted in over 4000 crocodiles being housed in this pen at the time of the principal investigator's visit to the farm, which made it impossible to identify and count diseased, vaccinated crocodiles. The determination of the extent of the outbreak in diseased crocodiles was further complicated by the slaughter of diseased crocodiles, already in progress at the time of the principal investigator's visit to the farm. Therefore, it was not possible to determine how many vaccinated crocodiles had already been slaughtered.

All the pens housing grower crocodiles were significantly overstocked at the time of the outbreak (building of additional pens was already in progress). The farm had also experienced very hot weather during the period preceding the outbreak. Both these factors could have significantly stressed the crocodiles, and increased their susceptibility to disease (Huchzermeyer 2003).

Logistics prevented performance of pathological examinations on study crocodiles throughout the trial period. However, necropsies were performed on diseased crocodiles during the time of the major disease outbreak in October 2011. A subset of diseased crocodiles and mortalities were examined, the number limited by time constraints. Five clinical cases (all severely affected and completely lame) and four mortalities (from the previous night) were examined. As stated, only four of these animals, two of the clinical cases and two of the mortalities, displayed clear signs of polyarthritis. Of the remaining crocodiles, the two remaining mortalities were too autolysed to make a diagnosis of cause of death and the three clinical cases displayed non-specific signs of disease, including generalized congestion which could indicate septicaemia.

Although the clinical signs and pathology were consistent with that expected for crocodile mycoplasmosis, culture was not performed to confirm the aetiology of this outbreak.

Despite all the mentioned technical difficulties, the managers of the crocodile farm felt that the vaccine did not provide protection against the disease in the face of an outbreak. The overall picture from the serological data, correlated with this conclusion.

SEROLOGICAL MONITORING

A significant part of the evaluation of vaccine efficacy was based on serological monitoring, because of the mentioned difficulties encountered with clinical and pathological monitoring. However, these are not without limitations, which will be discussed in this section.

The serological results of samples collected during the outbreak are presented in Tables 4.3 and 4.4, and Figures 4.9 and 4.10. As indicated, no correlation was found between vaccination status, disease status and serological status. Some explanations for these disappointing results, which indicate failure

of the vaccine, will be discussed. (As the same serological assays were employed, the limitations mentioned under “Immunogenicity” and “Performance of the latex agglutination assay” need to be considered but will not be repeated.)

The presence of antigen variation is the first and, probably, most important reason to consider for the failure of this *Mycoplasma* vaccine. As discussed in chapter 2, it is well recognized that many (if not all) pathogenic *Mycoplasma* species make use of variable immuno-dominant surface proteins and this has been presented as one of the primary reasons for the persistence of *Mycoplasmas* in the face of a specific host immune response (Bercina *et al.* 1994, Levisohn & Kleven 2000, Citti *et al.* 2010). Therefore, if the host is unable to eliminate the organism or to develop lasting immunity after a natural infection, it seems unlikely that an artificial method of immune-protection, mimicking natural infection, would provide complete, long-term protection (Razin 2006).

Inactivated vaccines face two additional limitations related to antigen variation. Firstly, it is likely that the surface antigens present in inactivated vaccines are fixed as the organism is grown and inactivated *in vitro*. Therefore, because the host immune system has only been primed with limited surface protein variation, it is even more likely that the invading *Mycoplasma* could simply express a different surface antigen and circumvent the pre-existing immune-protection. Secondly, it is unknown if the surface antigens expressed *in vitro* are representative of that expressed *in vivo*. The possibility of different proteins expressed in different milieu (which is well described for poultry mycoplasmosis) requires further investigation.

In addition to the above-mentioned disadvantages of inactivated vaccines, the composition of the vaccine also needs to be considered when apparent vaccine failure is reported. Inactivated vaccines are composed of two major components, namely the antigen and the adjuvant. There are various problems which could be encountered with the antigen, but, high antigen yield and good antigenicity is “the most important characteristics” (OIE 2008a). The general recommendation for poultry mycoplasmosis and CBPP is a titre above 10^8 CFU/ml (OIE 2008a, OIE 2008b). Although the titre of the antigen prepared for this vaccine was above this level (10^9 CFU/ml), the inherent immunogenicity of the *M. crocodyli* is unknown, and it is therefore difficult to extrapolate. Investigation of the inactivation process may be warranted as denaturation of proteins at the high levels of formalin and prolonged incubation period could have influenced the immunogenicity of the vaccine.

The role of the aluminium hydroxide adjuvant used in the experimental vaccine should also be considered. Aluminium salts have a long history of inclusion as vaccine adjuvants; in 1926 aluminium potassium sulphate was the first recorded vaccine adjuvant (Garçon *et al.* 2011). Since then aluminium salts, particularly aluminium hydroxide and aluminium phosphate, commonly called alums, have become the most commonly used vaccine adjuvants in both human and veterinary vaccines (Gupta 1998, Bowersock & Martin 1999, Aguilar & Rodríguez 2007). The success of alum adjuvants are primarily linked to their safety record (Bowersock & Martin 1999, Singh & O’Hagan 2003, Reed *et*

al. 2008, Garçon *et al.* 2011). Other advantages are the low cost (Bowersock & Martin 1999) and simple formulation, making it suitable for large scale production (Reed *et al.* 2008). However, it is also well known that alum does not stimulate cell-mediated immune responses, specifically Type I helper T-lymphocytes and Cytotoxic T-lymphocytes (Bowersock & Martin 1999, Aguilar & Rodríguez 2007, Reed *et al.* 2008, Garçon *et al.* 2011). This response is of critical importance in host-protection against intracellular pathogens, such as viruses. Other problems with alums include granulomas reported at the injection site (Reed *et al.* 2008) particularly if the vaccine is administered intradermally or subcutaneously (Aguilar & Rodríguez 2007) and loss of potency when vaccines are inadvertently frozen (Reed *et al.* 2008). Although good results are reported for inactivated *M. hyopneumoniae* (swine enzootic pneumonia) in alum adjuvant, results with human and poultry inactivated alum-adjuvanted *Mycoplasma* vaccines are quite disappointing (Linchevski *et al.* 2009, and better results are reported in poultry where mineral-oil adjuvants are used (Panigraphy *et al.* 1981). It may be useful to consider alternative vaccine adjuvants in future.

Given the mentioned restrictions and possible flaws of inactivated *Mycoplasma* vaccines, it seems questionable how any of these could have beneficial results, even though they are extensively used for certain diseases. It has to be considered that (as indicated in chapter 2) improved production and a reduction in the severity of clinical disease, rather than prevention of clinical disease in the face of virulent pathogen challenge, is the main advantage reported for most of the successful inactivated vaccines (Razin 2006). These advantages are linked, in general, to the presence of systemic immunity to *Mycoplasma*, which is more readily stimulated by administration of parenteral vaccines, in contrast to the local response, required to protect against host colonization etc., stimulated by administration of live vaccines to mucosal surfaces (Razin 2006). Therefore, it should be emphasized that, based on our current knowledge, a combination of live and inactivated vaccines are required to counteract all the effects of *Mycoplasma* infection.

Apart from problems inherent to the vaccine itself, one has to consider the method of vaccine evaluation. A major pitfall for the use of serology to monitor the level of protection, particularly for mycoplasmosis, is because it has been well documented that, at least in poultry, the levels of circulating antibody to *Mycoplasma* does not necessarily correlate with host protection (Lam & Lin 1984, Lin & Kleven 1984, Talkington & Kleven 1985, Whithear *et al.* 1990). It is possible that this discrepancy is due to the importance of cell-mediated immunity and/or also linked with surface antigen variation. Nonetheless, circulating antibody does play a role in resistance, with faster clearance of the organism and less severe tracheal lesions associated with the presence of antibody (Yagihashi & Tajima 1986, Elfaki *et al.* 1992, Yagihashi *et al.* 1992, Avakian & Ley 1993), and it is more practical to evaluate *in vitro*. Therefore, the use of serological assays are warranted, but these limitations need to be considered.

In conclusion, it can be stated that the performance of the vaccine in the face of a virulent disease outbreak was disappointing but there are several characteristics of the organism and disease in question, which could be responsible for the lack in protection.

Performance of the latex agglutination assay

The four performance characteristics evaluated were the sensitivity, specificity, and positive and negative predictive value of the latex agglutination test. The results are summarized in chapter 3. Unfortunately, the measured characteristics revealed disappointingly poor performance of the LAT, which need to be further analysed before conclusions and recommendations can be made.

Before analysing the performance of an assay, it is critical to reconsider the intended usage of the assay (Greenhalgh 1997, Banoo *et al.* 2010, OIE 2010). In this case, the LAT was initially developed as pen-side screening assay to be used by farmers/veterinarians in rural areas to confirm a diagnosis of *Mycoplasma*-associated arthritis. The ease and simplicity make it ideal for a pen-side assay but the poor performance characteristics require attention before it can be recommended.

The performance characteristics of a new assay are often compared to the performance of existing assays. Two other serological assays have been developed for crocodile mycoplasmosis, namely an indirect ELISA (Dawo & Mohan 2007) and a Western blot assay (Dawo and Mohan 2008), and both reported moderately-high sensitivity (above 80%) and very high specificity (100%). The LAT performs poorly in comparison to these assays. However, it has to be stated that the performance of these assays were evaluated with very small sample sizes and the “gold standard” in these cases were the presence or absence of clinical disease, which is not ideal.

Several technical difficulties need to be acknowledged. The evaluation of the performance of the latex agglutination assay was not included in the initial study plan, and resulted because the absence thereof was recognized as a constraint for interpretation of the serological results of the vaccination trial. Hence, important recommended elements were absent including identification of a suitable study population and study subjects, determination of the ideal sample size and reference test or tests (Banoo *et al.* 2010).

Indeed, the performance of the metabolic inhibition assay, used as the gold standard, has not been evaluated. Because it is prescribed as serological assay to differentiate *Mycoplasma* species, it is accepted that this assay is highly specific (Black 1973, Whitcom *et al.* 1995, Brown *et al.* 2007). However, the sensitivity of this type of assay has been questioned (Lin & Kass 1974). This was, however, the only other assay available.

Despite the mentioned problems and their potential complicating effects, the poor assay performance requires attention. Although poor specificity is not uncommon for a *Mycoplasma* agglutination assay,

the reported sensitivity is unusually poor – a sensitivity of 100% is reported for some of the serum plate agglutination assays used for *M. gallisepticum* in poultry (Ahmad *et al.* 1988).

Firstly, because of the uncertainties existing in our current understanding of reptile immunology, non-specific reactions due to natural antibodies or other unknown innate factors have to be considered (see immunogenicity above for further clarification). It will only be possible to reduce such confounding factors when they have been identified.

Secondly, the mentioned surface antigen variation has to be considered. Many assays, including agglutination assays and ELISA's, make use of pre-expressed antigens fixed on a solid phase. Therefore, if the host antibody response that is measured is predominantly aimed against a different antigen phenotype than used in the assay, it is very likely that false negative results could be reported. There is a distinct possibility that this complication could have an influence on the serological results during the disease outbreak (although it would not affect the serological results evaluated in the immunogenicity stage as the vaccine and the assay was prepared from the same isolate and culture conditions) as the identity of the strain/s causing the October 2011 outbreak, and their similarity to the strain used for preparation of the assays, is unknown.

In addition to these problems with the antigen, the possibility of severe denaturation also needs to be considered because of the, relatively, harsh inactivation conditions used in preparation of the antigen. Although the need thereof, from a biosafety point of view, can be appreciated, the possibility of the significant structural derangements needs to be considered, which could also contribute to the poor sensitivity recorded.

Another possible reason for the poor performance of the LAT is that optimization of assay conditions has not been performed. In particular, the optimal concentration of antigen and dilution of antiserum has not been determined. Although not commonly specified, it has been stated that a pro-zone effect, similar to that described for ELISA's, can occur in agglutination assays (Stanley 2002). In this zone, optimal agglutination would be inhibited by an overabundance of antibody. Dilution of the serum (antibody) would result in an increase in visible agglutination, and improvement of the tests' performance.

In conclusion, although the latex agglutination assay was less complex and time consuming to perform and did not require any advanced equipment, the poor performance reported in this trial indicates that further sophistication of the assay is warranted before it can be recommended for routine use.

CHAPTER 6: CONCLUSION

The serological analysis and outcome of the disease challenge indicated that the vaccine did not stimulate protective immunity. Evaluation of vaccine efficacy was however, hampered by a multitude of complications and limitations. Various reasons for the poor performance of the vaccine should be considered. These include aspects of *Mycoplasma* pathogenicity, including surface antigen variation and the interaction between the pathogen and the host immune system, exposure of animals to the pathogen prior to vaccination, vaccine formulation and overwhelming infection. The parameters used to evaluate vaccine efficacy also need consideration as various constraints have been described for *Mycoplasma* sero-monitoring and, from reports on mycoplasmosis in other species, inactivated vaccines are more reliable in reducing disease severity than to prevent disease. The predisposing factors surrounding the outbreak of mycoplasmosis also need investigation, as these could play an important role in breakdown of immune-protection.

The latex agglutination assay performed poorly in comparison to other serological assays for crocodile *Mycoplasma*. Various reasons for the poor performance can be suggested, including the unique host and our lack of knowledge on its immune system, lack of assay optimization and the inherent constraints of the assay. It is suggested that this assay should not be used as diagnostic assay without confirming the results with another assay, such as culture.

The trial emphasized the need for further research in a variety of areas. Firstly, the knowledge of reptile immunology, and specifically crocodile immunology, is deficient in comparison to mammalian and even avian immunology. This makes the development and evaluation of applied procedures, such as vaccination trials and sero-diagnostics, very challenging. Secondly, the epidemiology of and predisposing factors to crocodile mycoplasmosis require urgent attention. Without better knowledge on the source of infection, transmission, possible disease reservoirs etc. development of a scientifically based control strategy is virtually impossible. Related to this, is the necessity for reliable and fast assays, such as PCR, to evaluate host infection with the pathogen. Furthermore, research on the characteristics of *Mycoplasma crocodyli* is required in order to determine the presence of, extent and influence of surface antigen variation and the host-parasite interaction on the disease outcome.

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