University of Nevada, Reno

Mycoplasma agassizii Infections in the Desert Tortoise (*Gopherus agassizii*)

A dissertation submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Cell and Molecular Biology

by

Hamid A. Mohammadpour

Dr. Kenneth Hunter/Dissertation Advisor

December, 2011

University of Nevada, Reno

Statewide • Worldwide

THE GRADUATE SCHOOL

We recommend that the dissertation prepared under our supervision by

HAMID A. MOHAMMADPOUR

entitled

***Mycoplasma Agassizii* Infections In Desert Tortoise (*Gopherus Agassizii*)**

be accepted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Kenneth W. Hunter, Sc.D, Advisor

Doug Redelman, Ph.D, Committee Member

David Lupan, Ph.D, Committee Member

David Aucoin, Ph.D, Committee Member

Richard C. Tracy, Ph.D, Graduate School Representative

Marsha H. Read, Ph. D., Associate Dean, Graduate School

December, 2011

ABSTRACT

Mycoplasma agassizii is a cause of upper respiratory tract disease in the threatened desert tortoise (*Gopherus agassizii*) of the Southwestern United States. Two technical challenges have impeded critical microbiological studies of this microorganism. First, its small size (<300 nm) limits the use of light microscopy for cell counting. Second, its slow growth in broth and agar cultures impedes colony counting. Our aim was to develop a rapid and sensitive flow cytometric method using a vital fluorescent dye to enumerate viable *M. agassizii* cells. We discovered that the non-fluorescent molecule 5-carboxyfluorescein diacetate acetoxymethyl ester penetrates *M. agassizii* cell membranes and is converted in the cytoplasm to the fluorescent molecule 5-carboxyfluorescein by the action of intracellular esterases. Labeled mycoplasma cells can be easily detected by flow cytometry, and cultures with fewer than 100 viable mycoplasma cells μl^{-1} can be labeled and counted in less than 1 h. Experiments using temperature-induced cell death demonstrated that this procedure labels only viable *M. agassizii* cells. This technique should facilitate basic immunological, biochemical, and pharmacological studies of this desert tortoise pathogen which may lead to new diagnostic and therapeutic methods.

To facilitate seroepidemiologic studies of infectious diseases caused by microorganisms like *M. agassizii*, isotype-specific antibody reagents are needed. Our goal was to prepare isotype-specific rabbit polyclonal antibodies to desert tortoise IgM and IgY. A 50% saturated ammonium sulfate precipitate of desert

tortoise serum was fractionated by Sephacryl A-300 gel filtration chromatography and IgM-rich and IgY-rich fractions were collected. SDS-PAGE confirmed the molecular weights of the heavy chains of tortoise IgM and IgY to be 71 kD and 68 kD respectively. Rabbits immunized with these separate fractions produced high titer sera that cross-reacted slightly with the opposite heavy chain and strongly with the conserved 22 kD light chain. To make heavy chain isotype-specific reagents, the immunoglobulin fraction of each antiserum was cross-absorbed using polystyrene microspheres coated with the opposite isotype. SDS-PAGE and Western blots confirmed the heavy chain specificity of the cross-absorbed antibodies. These high avidity rabbit polyclonal antibody reagents were used in ELISA and Western blots to document the primary and secondary isotype-specific antibody responses to *M. agassizii* infection and ovalbumin immunizations in desert tortoises. The availability of rabbit polyclonal antibodies to desert tortoise IgM and IgY will allow more detailed studies of the humoral immune response of the desert tortoise to microbial pathogen.

ACKNOWLEDGMENTS

I am greatly indebted to a number of individuals whose expertise and assistance has been instrumental in guiding me through the completion of my studies. First, I would like to express my sincere gratitude and appreciation to Dr. Kenneth Hunter who became my role model and mentor. He always encouraged me and remained enthusiastic about my work. He provided suggestions, strategies, and focus that were important to the completion of my projects and formed the foundation my studies. His leadership, along with his optimism, was unequivocal**.** I would also like to thank the other members of my graduate committee.

Dr. Doug Redelman for his encouragement and support as well as his direct efforts toward the completion of the flow cytometry method for the enumeration of viable mycoplasma. His expertise was invaluable and I will always be grateful.

Dr. C. Richard Tracy, who truly is on a mission to save Nevada's wildlife, particularly those who need the most help, such as the desert tortoises.

Dr. Mark Hall, who I have known and worked with many years, was always there to lend a helpful hand and his encouragement led me into the graduate program.

Dr. David Lupan, who has been gracious with his time away from his busy work schedule. His dedication to not only graduate education but to the advancement of the medical school is admirable. Also, I would like to thank Dr.

Dave AuCoin for his participation on my committee. His prompt agreement to serve on my committee was greatly appreciated.

I would like to extend my sincere appreciation to Dr. Sally duPre' for her assistance and support. I am also grateful to Dr. Bill Andrews and Sierra Sciences, LLC for their steadfast support of my efforts. There were many others who were instrumental to my work and I would like to extend my appreciation to them all. In particular I would like to thank Dr. Sandmeier and Nicole Maloney as well as John Gray for his excellent care of the desert tortoises in our vivarium.

Finally I would like to thank Dr. Laura Briggs who I have known and worked with for a long time. She always gives me encouragement and support and tries to correct my English where needed most, especially during the preparation of this manuscript. I have yet to meet anybody that I can work with so professionally and effectively. Her analytical and technical skill, in addition to her personal friendship is irreplaceable.

All research is in need of financial support, and I owe a sincere debt of gratitude to the Clark County, Nevada Desert Conservation Program and the Desert Tortoise Recovery Office of the U.S. Fish and Wildlife Service for their financial support in conducting this project. I am also thankful for the financial support I received from the Crane Biomedical Research Scholarship program through the University of Nevada, School of Medicine.

TABLE OF CONTENTS

List of ABBREVIATIONS

ATCC: American Type Culture Collection

BCA: bicinchoninic acid protein assay

5-CFDA-AM: carboxyfluorescein diacetate acetoxymethyl ester

5-CF: 5-carboxyfluorescein

CMRL-1066: Connaught Medical Research Laboratories

DMSO: dimethyl sulfoxide

EDTA: ethylenediaminetetraacetic acid

PBS: phosphate buffered saline

RaTIgM: Rabbit anti-Tortoise IgM

RaTIgY: Rabbit anti-Tortoise IgY

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

PBS-T: phosphate buffered saline (PBS) solution with the detergent Tween 20

SP4: Spiroplasma Medium 4

TBS-T: tris-buffered saline and Tween 20

TMB: tetramethylbenzidine

URTD: upper respiratory tract disease

List of FIGURES

Chapter 1

Chapter 2

Chapter 3

Supplemental Data

Chapter One

Introduction

Decline in wild populations of the mojave desert tortoise (*Gopherus agassizii*)

The Mojave Desert tortoise is native to the southwestern United States (Fig. 1). These extraordinary animals are adapted to arid habitats characterized by prolonged drought, wide temperature fluctuations, and food shortages. The desert tortoise is able to live where ground temperatures may exceed 140º F in the summer and freezing in the winter because they dig and reside in underground burrows (Van Devender, 2006). Desert tortoises possess unique physiologic processes to deal with dehydration, excess nitrogenous wastes, and high salt loads (Dantzler and Schmidt-Nielsen, 1966; Nagy and Medica, 1986; Peterson, 1994; Nussear *et al*., 2007). Although desert tortoises can live to be more than 50 years old in the wild (Germano, 1992), encroachment of civilization on their habitat, together with periods of severe drought, have placed significant stress on these ancient reptiles. There is evidence that populations of the Mojave Desert tortoise have declined over the last three decades (Corn, 1994; Sandmeier *et al*., 2009). These population declines had been attributed to a variety of factors, including drought (Longshore *et al*., 2003; Peterson, 1996), and the impacts of human activity, predation, and habitat loss. However, whenever precipitous declines in wild animal populations are observed, infectious diseases need to be considered as a potential cause. In 1990 the desert tortoise was listed as threatened under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service, 1994), partly because many desert tortoises had been observed with a clinical condition that is now identified as upper respiratory tract disease (URTD).

Figure 1. Desert Tortoise (*Gopherus agassizii)* in the wild. Photograph taken by Dr. Fran Sandmeier**.**

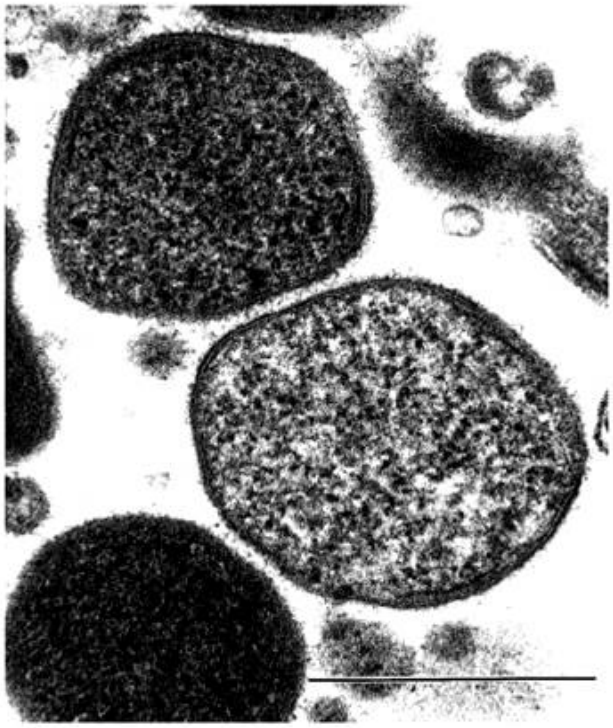

Figure 2. Electron micrograph of *Mycoplasma agassizii* (Brown *et al*., 2001). Bar, 0.5 µM.

URTD in the mojave desert tortoise

The symptom complex of URTD in tortoises includes nasal exudates, swelling around the eyes (palpebral edema), and histological lesions in the nasal epithelium and in the mucosa of the upper respiratory tract (Brown *et al*., 1994; Christopher *et al*., 2003). Lethargy and death have been observed in severe cases. URTD was first noticed in desert tortoises that harbored the Gram-negative bacterium *Pasteurella testudinis* (Snipes and Fowler, 1980), and later the bacterium *Mycoplasma agassizii* was found in desert tortoises with URTD (Jacobson *et al*., 1991; Schumacher *et al*., 1993; Brown *et al*., 1994). Studies of *M. agassizii* revealed that this microorganism fulfilled Koch's postulates as an etiologic agent of URTD (Brown *et al*., 1994). A related species called *M. testudineum* has also been implicated as a cause of URTD (Brown *et al*., 2004). More recently, a herpes virus has been identified in desert tortoises with URTD (Pettan-Brewer *et al*., 1996; Johnson *et al*., 2005). Despite evidence that several microorganisms may cause URTD, the vast majority of the research over the last twenty years has focused on *M. agassizii* (Sandmeier *et al*., 2009).

Small size and slow growth in broth and agar cultures has impeded research on *Mycoplasma agassizii*

M. agassizii is a bacterium that belongs to the class Mollicutes (Barile *et al.*, 1985). This unique group of bacteria is characterized by the lack of a cell wall and plasma membranes that contain sterols. Mycoplasma are small in physical size, and they possess a small genome (Blanchard and Browning, 2005).

Evolutionarily, they likely evolved from Gram-positive bacteria by reductive evolution making them possibly the smallest self-replicating organisms found in nature (Razin *et al*., 1998). These bacteria can cause acute and chronic diseases in humans, animals, insects and plants.

M. agassizii is a pleiomorphic mycoplasma with an average diameter of less than 300 nm, thus it cannot be visualized by light microscopy (Fig. 2). These bacteria are extremely slow growing even in highly enriched broth cultures (Hunter *et al*., 2008). Historically, researchers have used color changing units in broth cultures as well as colony forming units on solid agar media to monitor and quantify other mycoplasma species in the laboratory. However, in broth cultures of *M. agassizii* it takes several days to visualize even slight changes in the color of the medium (color changing units) which could be the result of many processes other than mycoplasma growth. Moreover, it takes up to 8 wk for even small colonies to appear on solid agar (Wendland *et al.,* 2007; Hunter *et al.,* 2008). The inability to determine the numbers of viable *M. agassizii* in broth cultures, and by colony counting on agar media, has significantly impeded studies of the immunology, biochemistry, and pharmacology of this microorganism.

Flow cytometry as an alternative method for quantifying viable *M. agassizii*

Flow cytometric methods for counting bacteria that provide faster results than culture methods have been developed previously (Lopez-Amoros *et al.,*

1995; Langsrud and Sundhein, 1996; Henningson *et al.,* 1998; Rattanasomboon *et al.,* 1999; Guasekera *et al.,* 2000; Holm and Jespersen 2003). Recently flow cytometry using the DNA intercalating fluorophore SYBR Green in combination with the membrane impermeant fluorophore propidium iodide compared favorably with colony counting for determining the numbers and viability of various mycoplasma species (Assunção *et al.,* 2005; Assunção *et al.,* 2006a; Assunção *et al.,* 2006b). Although successful as a cell counting and viability determining tool, the present methods requires the use of two fluorophores.

Enumerating viable *M. agassizii* using a single fluorophore method

The development of an alternative flow cytometric method that employs a single fluorophore for both identifying and enumerating viable *M. agassizii* cells in broth culture was the first focus of our research. We investigated the use of carboxyfluorescein diacetate acetoxymethyl ester (5-CFDA-AM) as a candidate single fluorophore. 5-CFDA-AM is a non-fluorescent, lipophilic substrate that enters cells by simple diffusion (Goodall and Johnson, 1982). Through a hydrolysis reaction mediated by esterases in viable cells, 5-CFDA-AM is converted to the fluorescent 5-carboxyfluorescein (5-CF), which is more polar and is released slowly from the cell. In the first study presented in this dissertation we demonstrate that *M. agassizii* possesses intracellular esterases that can generate 5-CF from 5-CFDA-AM, and that these fluorescent molecules remain in the cell and can be used to detect and count viable *M. agassizii* by

flow cytometry (note that this work has been published; *Mohammadpour, H., duPre', S.A., Tracy, C.R., Redelman, D., and Hunter K.W. 2010. Flow cytometric method for quantifying viable Mycoplasma agassizii, an agent of upper respiratory tract disease in the desert tortoise (Gopherus agassizii), in broth culture.* Lett. Appl. Microbiol. **50**, *347-351*).

Seroepidemiological studies of *M. agassizii* infections in desert tortoises

Understanding the prevalence of *M. agassizii* infections in the desert tortoise was immediately identified as critical for the management of these animals. The logistical difficulties of capturing wild desert tortoises and culturing *M. agassizii* and other URTD pathogens from their upper respiratory tracts led to a reliance on antibody-based methods for identifying infected tortoises (Sandmeier *et al*., 2009). Schumacher *et al*. (1993) described a monoclonal antibody-based enzyme linked immunoassay (ELISA) for measuring the antibody response to *M. agassizii* in the serum of desert tortoises. This method, which became the gold standard, used a monoclonal antibody raised in mice against the light chain of the tortoise immunoglobulin molecule. Earlier studies of chelonians (Benedict and Pollard, 1972; Leslie and Clem, 1972; Ambrosius, 1976) had identified three major immunoglobulin classes (IgM, IgY, and IgYΔFc). Herbst and Klein (1995) reported similar findings for the green sea turtle (*Chelonia mydas*), and Schumacher *et al*. (1993) reported similar findings in the desert tortoise. Although it was known that there are multiple immunoglobulin

light chain isotypes in other species (Origgi, 2007) it was presumed that the light chain of the desert tortoise was conserved among the major immunoglobulin isotypes. Using this ELISA, a series of seroepidemiological studies were performed that identified up to 20% of desert tortoises as “infected” with *M. agassizii* (Jacobson *et al*., 1995; Lederle *et al*., 1997; Schumacher *et al*., 1997; Brown *et al*., 1999b; Christopher *et al*., 2003; Dickinson *et al*., 2005). Despite the fact that antibody-based methods are at best surrogates for identifying desert tortoises that are colonized or actively infected with *M. agassizii*, for several years tortoises deemed to be serologically positive were euthanized to prevent the spread of *M. agassizii* among desert tortoise populations, particularly populations of tortoises that had been captured for translocation to new habitats (Jacobson *et al*., 1995; Sandmeier *et al*., 2009). In 2008, our laboratory reported the development of a rabbit polyclonal antibody reagent that reacted with the heavy and light chains of all desert tortoise immunoglobulin isotypes (Hunter *et al*., 2008). This reagent was used to test a group of egg-reared desert tortoises that had neither lived in the wild nor been exposed to other wild tortoises. Using both ELISA and the more discriminatory Western blot method, it was discovered that non-colonized desert tortoises have natural antibodies to *M. agassizii*. Natural antibodies have been identified in humans and non-human vertebrates (Avrameas, 1991; Coutinho *et al.,* 1995; Boes, 2000), are predominantly of the IgM class, and represent a component of innate immunity (Boes *et al*., 1998; Baumgarth *et al*., 2005; Ochsenbein and Zinkernagel, 2000). The rabbit polyclonal reagent was also used to test sera from desert tortoises at the Desert

Tortoise Conservation Center in Clark County, NV that had previously tested positive by the monoclonal antibody-based ELISA. Many of the tortoises that were deemed to be positive by the monoclonal ELISA at a level that would trigger euthanasia showed a natural antibody pattern on Western blot using the polyclonal reagent. Moreover, ELISA titers of the presumed positive tortoises overlapped with titers of some non-infected tortoises, calling into question the selection of a cut-off point for identifying a tortoise as serologically positive for *M. agassizii*. These studies informed a decision to halt the use of ELISA data to determine the fate of captured desert tortoises (Sandmeier *et al*., 2009). To determine whether a desert tortoise is infectious and capable of transmitting *M. agassizii* to other tortoises, a microbiological method is the better choice. Recently, our laboratory published a quantitative polymerase chain reaction (qPCR) method for identifying *M. agassizii* DNA in nasal lavage fluid (Dupre' *et al*, 2011).

Understanding the natural history of *M. agassizii* infections in the desert tortoise

Antibody-based methods still provide important information, and could provide even more important information if the natural history of *M. agassizii* infections in desert tortoises was better understood. There is no doubt that very high titers of antibodies, determined by monoclonal or polyclonal ELISA methods, are indicative of a past infection with *M. agassizii*. The Western blot banding pattern can further distinguish non-infected tortoises with natural antibodies from

tortoises that have clearly made an adaptive immune response to the pathogen. However, without knowing when a particular desert tortoise was exposed to *M. agassizii*, it is impossible to know how long it takes for a tortoise to develop an antibody response (e.g., seroconversion). Moreover, the evolving antibody response to specific antigenic components of *M. agassizii* is not known, nor the persistence of antibodies over time. In addition, it is known from studies of the mammalian immune system that the initial immunoglobulins produced following infection are predominantly of the IgM class, and that B cells producing these antibodies can undergo isotype switching to other immunoglobulin classes (Flajnik, *et al*., 2003). In humans and other mammals, the detection of predominantly IgM antibodies in the serum is indicative of an early infection, and the presence of other classes or isotypes like IgG suggests a more protracted infection (Abbas *et al*., 1991).

As mentioned previously, chelonians are thought to have two major immunoglobulin isotypes known as IgM and IgY, and a truncated version of IgY called IgY (ΔFc) that is missing the two carboxy terminal heavy chain domains (Benedict and Pollard, 1972; Leslie and Clem, 1972; Origgi, 2007). Tortoise IgM and IgY resemble mammalian IgM and IgG respectively (Ambrosius, 1976). IgY is considered to be the functional counterpart of mammalian IgG and IgE (Warr *et al.,* 1995). According to Schumacher *et al.* (1993), the molecular size of the light and heavy chain of *Gopherus agassizii* IgY are 27 and 65 kDa respectively. Tortoise IgM is a pentameric molecule with a presumed molecular weight of approximately 950 kDa, and heavy chains of approximately 75kD.

Unlike the commercially available reagents for detecting the various immunoglobulin isotypes of human, mice, and many other species, there are no immunological reagents that can distinguish between the two major immunoglobulin isotypes of the desert tortoise. We felt that such reagents would provide more robust information about the antibody response to *M. agassizii* in the desert tortoise. In the second part of this dissertation, the development of IgM and IgY-specific rabbit polyclonal antibodies is described. Using our flow cytometric method (Mohammadpour *et al*., 2010) to prepare the inocula of viable bacteria, we infected six laboratory-housed desert tortoises with known numbers of *M. agassizii*, and we used our isotype-specific tortoise antibody reagents to follow the evolving humoral immune response.

RESEARCH OBJECTIVES

The overall goal of this research was to develop more reliable methods for studying *M. agassizii* and other infectious diseases in the desert tortoise. Our first objective was to develop a flow cytometric method to quantify viable *M. agassizii* using carboxyfluorescein diacetate acetoxymethyl ester (5-CFDA-AM), a non-fluorescent esterified substrate which is converted to a fluorescent 5-carboxyfluorescein (5-CF) in viable mycoplasma. In **Chapter Two** we describe the development of this technique, and demonstrate its use to determine the growth characteristics of *M. agassizii* in broth culture. This work paves the way for future biochemical, pharmacological, and immunological studies of this microorganism.

Our second objective was to prepare isotype-specific antibodies to the major immunoglobulins of the desert tortoise. In **Chapter Three** we describe the use of cross-absorption to generate IgM and IgY heavy chain-specific rabbit polyclonal antibody reagents to facilitate the study of humoral immune responses in the desert tortoise. To demonstrate the utility of the isotype-specific reagents, two studies were performed using egg-reared, laboratory-housed desert tortoises. The first study used ovalbumin immunization to document an early but modest IgM response that was followed by a more robust IgY response to this non-replicating foreign antigen. A second study was performed to examine the isotype-specific immune response to the infectious agent, *M. agassizii*. Using the flow cytometric method described in Chapter Two, we prepared a viable

inoculum of *M. agassizii* and infected six laboratory-housed desert tortoises. We used the isotype-specific reagents together with ELISA and Western blot to follow the humoral immune response to *M. agassizii*.

References

Abbas, A.K., Lichtman, A.H., Pober, J.S. (1991). *Cellular and Molecular Immunology*. W.B. Saunders Co, Philadelphia, PA., p. 188.

Ambrosius, H. (1976). Immunoglobulins and antibody production in reptiles, in *Comparative Immunology*, Marchalonis, J.J (Ed.), Academic Press, New York, 298-334.

Assunção, P., Diaz, R., Comas, J., Ruiz de Galarreta, C.M., Llamazares, O.G. and Poveda, J.B. (2005). Evaluation of *Mycoplasma hyopneumoniae* growth by flow cytometry. *J. Appl. Microbiol.* **98**, 1047-1054.

Assunção, P., Antunes, N.T., Rosales, R.S., de la Fe, C., Poveda, C., Poveda, J.B and Davey, H.M. (2006a). Flow cytometric method for the assessment of the minimal inhibitory concentrations of antibacterial agents to *Mycoplasma agalactiae*. *Cytometry* **69**, 1071-1076.

Assunção, P., Antunes, N.T., Rosales, R.S., Poveda, C., Poveda, J.B and Davey, H.M. (2006b). Flow cytometric determination of the effects of antibacterial agents on Mycoplasma agalactiae, Mycoplasma putrefaciens, Mycoplasma capricolum subsp. capricolum, and Mycoplasma mycoides subsp. mycoides large colony type. *Antimicrob Agents Chemother* **50**, 2845-2849.

Avrameas, S. (1991). Natural autoantibodies: from ‘horror autotoxicus’ to ‘gnothi seauton. *Immunol. Today* **12**, 154-159.

Barile, M.F., Razin, S., Tully, J.G., Whitcomb, R.F. (Eds.), 1985. The Mycoplasmas. Academic Press, Inc., Orlando.

Baumgarth, N., Tung, J.W., Herzenberg, L.A., (2005). Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. *Springer Semin. Immunopathol.* **26**, 347-362.

Benedict, A.A. and Pollard, L.W. (1972). Three classes of immunoglobulins found in the sea turtle, *Chelonia mydas*. Folia Microbiol. **17**, 75–78.

Blanchard, A. and Browning, G., (2005), *Mycoplasma: molecular biology, pathology and strategies for control,* CRC Press, Inc

Boes, M., Prodeus, A.P., Schmidt, T., Carroll, M.C., Chen, J. (1998). A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. J. *Exp. Med*. **188**, 2381-2386.

Boes, M., (2000). Role of natural and immune IgM antibodies in immune responses. *Mol. Immunol.* **37**, 1141-1149.

Brown, D.R., Merritt, J.L., Jacobson, E.R., Klein, P.A., Tully, J.G., and Brown, M.B. (2004). *Mycoplasma testudineum* sp. nov., from a desert tortoise (*Gopherus agassizii*) with upper respiratory tract diseases. *Inter. J. Syst. Evol. Microbiol.* 54, 1527-1529.

Brown, M.B., McLaughlin ,G.S., Klein, P.A., Crenshaw, B.C., Schumacher, I.M., Brown, D.R., and Jacobson, E.R. (1999). Upper respiratory tract disease in the gopher tortoises is caused by *Mycoplasma agassizii*. *J. Clin. Microbiol.* **37**, 2262-2269.

Brown, M.B., Brown, D.R., Klein, P.A., McLaughlin, G.S, Schumacher, I.M., Jacobson, E.R., Adams, H.P. and Tully, J.G. (2001). *Mycoplasma agassizii* sp. nov., isolated from the upper respiratory tract of the desert tortoise (*Gopherus agassizii*) and the gopher tortoise (*Gopherus polyphemus*). *Int. J. Syst. Evol. Microbiol.* **1,** 413-418.

Christopher, M.M., Berry, K.H., Henen, B.T., and Nagy, K.A. (2003). Clinical disease and laboratory abnormalities in free-ranging tortoises in California (1990–1995). *J. Wildlife Dis.* **39**, 35-56.

Corn, P.S., 1994. Recent trends of desert tortoise populations in the Mojave desert. In: Bury, R.B., Germano, D.J. (Eds.), Biology of North American Tortoises, Fish and Wildlife Research, vol. 13. US Department of the Interior, National Biological Survey, Washington, DC, pp. 85–93.

Coutinho, A., Kazatchkine, M.D., Avrameas, S. (1995). Natural autoantibodies. *Curr. Opin. Immunol*. **7**, 812-818.

Dantzler W.H, Schmidt-Nielsen, B. (1966). Excretion in fresh water turtle (*Pseudemys scripta*) and desert tortoise (*Gopherus agassizii*). *Am. J*. *Physiol*. **201**,198-210.

Dickinson, V.M., Schumacher, I.M., Jarchow, J.L., Duck, T., Schwalbe, C.R., (2005). Mycoplasmosis in free-ranging desert tortoises in Utah and Arizona. *J. Wildlife Dis.* **41**, 839-842.

Dupre', S.A., Tracy, C.R., Hunter, K.W. (2011). Quantitative PCR method for detection of mycoplasma spp. DNA in nasal lavage samples from the desert tortoise (*Gopherus agassizii*). *J. Microbiol. Methods* **86**(2), 160-165.

Flajnik, M.F., Miller, K. and du Pasquier, L. (2003) In: Paul, W.E. (Ed.), Fundamental Immunology. Lippincott Williams &Wilkins, Philadelphia, 519–570.

Germano, D.J. (1992). Longevity and age-size relationships of populations of desert tortoises. *Copeia*, **2**, 367-374.

Goodhall, H. and Johnson, M.H. (1982). The use of carboxyfluorescein diacetate to study formation of permeable channels between mouse blastomeres. *Nature* **295**, 524-526.

Gunasekera T.S., Attfield, P.V. and Veal, D.A. (2000). A flow cytometry method for rapid detection and enumeration of total bacteria in milk. *Appl. Environ. Microbiol* . **66**, 1228-1232.

Henningson, E.W., Corcova, Z., Sandstrom, G. and Forsman, M. (1998). Flow cytometric assessment of the survival ratio of *Francisella tularensis* in aerobiological samples. *FEMS Microbiol. Ecol* . **25**, 241-249.

Herbst , L.H., and Klein, P.A. (1995). Monoclonal antibodies for the measurement of class-specific antibody responses in the green turtle, *Chelonia mydas*. *Vet. Immunol. Immunopathol.* **46**, 317-335.

Holm, C. and Jespersen, l. (2003). A flow-cytometric gram-staining technique for milk-associated bacteria. *Appl. Environ. Microbiol* . **69**, 2857-2863.

Hunter, K.W., duPré, S.A., Sharp, T., Sandmeier, F.C and Tracy, C.R. (2008). Western blot can distinguish natural and acquired antibodies to *Mycoplasma agassizii* in the desert tortoise (*Gopherus agassizii*). *J. Microbiol. Meth.* **75**, 464-471.

Jacobson, E.R., Gaskin, J.M., Brown, M.B., Harris R.K., Gardiner C.H., LaPointe J.L., Adams, H.P. and Reggiardo C. (1991). Chronic upper respiratory tract disease of free-ranging desert tortoises (*Xerobates agassizii*). *J. Wildl. Dis.* **27,** 296-316.

Jacobson, E.R., Brown, M.B., Schumacher, I.M., Collins, B.R., Harris, R.K., Klein, P.A. (1995). Mycoplasmosis and the desert tortoise (*Gopherus agassizii)* in Las Vegas Valley, Nevada. *Chelonian Conserv. Biol.* **1**, 279-284.

Johnson, A.J., Pessier, A.P., Wellehan, J.F.X., Brown, R., Jacobson, E.R. (2005). Identification of a novel herpesvirus from a California desert tortoise (*Gopherus agassizii*). *Vet. Microbiol.* **111**, 107-116.

Langsrud, S. and Sundheim, G. (1996). Flow cytometry for rapid assessment of viability after exposure to a quaternary ammonium compound. *J. Appl. Bacteriol.* **81**, 411-418.

Lederle, P.E., Rautenstrauch, K.R., Rakestraw, D.L., Zander, K.Z., Boone, J.L., 1997. Upper respiratory tract disease and mycoplasmosis in desert tortoises from Nevada. *J. Wildl. Dis.* **33**, 759-765.

Leslie, G.A. and Clem, L.W. (1972) .Phylogeny of immunoglobulin structure and function VI. 17S, 7.5S, and 5.7s anti-DNP of the turtle, *Pseudemys scripta*. *J. Immunol.* **108**, 1656-1664.

Longshore, K.M., Jaeger, J.R., Sappington, J.M. (2003). Desert tortoise (*Gopherus agassizii*) survival at two eastern Mojave Desert sites: death by short-term drought? *J. Herpetol.* **37**, 169-177.

Lopez-Amoros, R., Comas, J. and Vivas-Rego, J. (1995). Flow cytometric assessment of *Escherichia coli* and *Salmonella typhimurium* starvation-survival in seawater using rhodamine 123, propidium iodide and oxonol. *Appl. Environ. Microbiol.* **61**, 2521-2526.

Mohammadpour, H.A., Tracy, C.R., Redelman, D., duPre,' S.A. and Hunter, K.W. (2010). Flow cytometric method for quantifying viable *Mycoplasma agassizii*, an agent of upper respiratory tract disease in the desert tortoise (*Gopherus agassizii*). *Lett. Appl. Microbiol.* **50**, 347-51.

Nagy, K.A., and Medica, P.A., (1985). Altered energy metabolism in an irradiated population of lizards at the Nevada Test Site. *Radiat. Res*. **103**, 98-104.

Nussear, K.E., Esque, T.C., Haines, D.F. and Tracy, C.R., (2007). Desert Tortoise hibernation: Temperatures timing, and environment *Copeia* **2**, Pages: 378-386**.**

Ochsenbein, A.F., and Zinkernagel, R.M., (2000). Natural antibodies and complement link innate and acquired immunity. *Immunol. Today* **21**, 624-629.

Origgi, F.C. 2007. Reptile Immunology, in *Infectious Diseases and Pathology of Reptiles*, Jacobson ER (Ed.), Taylor and Francis Group, Boca Raton, Florida, 131-157.

Peterson, C.C. (1994). Different rates and causes of high mortality in two populations of the threatened desert tortoise *Gopherus agassizii*. *Biological Conserv.* **70**, 101-108.

Peterson, C. C. (1996). Anhomeostasis: Seasonal water and solute relations in two populations of the desert tortoise (*Gopherus agassizii)* during chronic drought. *Physiol. Zool,* **69,** 1324-1358.

Pettan-Brewer, K.C.B., Drew, M.L., Ramsay, E., Mohr, F.C. and Lowenstine, L.J. (1996). Herpesvirus particles associated with oral and respiratory lesions in a California desert tortoise (Gopherus agasszii). *J. Wildl. Dis.* **32**, 521-526.
Rattanasomboon, N., Bellara, S.R., Harding, C.L., Fryer, P.J., Thomas, C.R., Al-Rubeai, M. and McFarlane, C.M. (1999). Growth and enumeration of the meta spoilage bacterium *Brochothrix thermosphacta*. *Inter. J. Food. Microbiol.* **51**, 145-158.

Razin, S., Yogev, D., Naot, Y. (1998). Molecular biology and pathogenicity of mycoplasmas. *Microbiol. Mol. Biol. Rev.* **62**, 1094-1156.

Sandmeier, F.C., Tracy, C.R., duPré, S., and Hunter, K.W. (2009). Upper respiratory tract disease (URTD) as a threat to desert tortoise populations: a reevaluation. Biol.Conserv. **142**, 1255-1268.

Schumacher, I.M., Brown, M.B., Jacobson, E.R., Collins, B.R. and Klein, P.A. (1993). Detection of antibodies to a pathogenic mycoplasma in desert tortoises (*Gopherus agassizii*) with upper respiratory tract disease. *J. Clin. Microbiol.* **31**, 1454-1460.

Schumacher, I.M., Hardenbrook, D.B., Brown, M.B., Jacobson, E.R., Klein, P.A., (1997). Relationship between clinical signs of upper respiratory tract disease and antibodies to *Mycoplasma agassizii* in desert tortoises from Nevada. *J. Wildl. Dis.* **33**, 261-266.

Snipes, K.P., Fowler, M.E. (1980). A Pasteurella sp. associated with respiratory disease in captive desert tortoises. *J. Amer. Vet. Med. Assoc.* **177**, 804-807.

U.S. Fish and Wildlife Service. (1994). Desert tortoise (Mojave population) recovery plan. U.S. Fish and Wildlife Service, Portland, Oregon, USA. 73 pp. plus appendices.
Van Devender, T.R. The Sonoran Desert Tortoise: Natural History, Biology, and Conservation, University of Arizona Press, Tucson, AZ, 2006, 288 p.

Warr G.W., Magor K.E., and Higgins, D.A. (1995). IgY: clues to the origins of modern antibodies. *Immunol. Today* **16**, 392-398.

Wendland, L.D., Zacher, L.A., Klein, P.A., Brown, D.R., Demcovitz, D., Littell, R. and Brown, M.B. (2007). Improved enzyme-linked immunosorbent assay to reveal *Mycoplasma agassizii* exposure: a valuable tool in the management of environmentally sensitive tortoise populations. *Clin Vaccine Immunol* **14,** 1190-1195.

Chapter Two

Flow cytometric method for quantifying viable *Mycoplasma agassizii*, an agent of upper respiratory tract disease in the desert tortoise (*Gopherus agassizii*)

Abstract

Mycoplasma agassizii is a cause of upper respiratory tract disease in the threatened desert tortoise of the Southwestern United States. Two technical challenges have impeded critical microbiological studies of this microorganism: its small size limits the use of light microscopy for cell counting, and it's extremely slow growth in broth and agar cultures impedes colony counting. Our aim was to develop a rapid and sensitive flow cytometric method using a vital fluorescent dye to enumerate viable *M. agassizii* cells.

Here we demonstrate that the non-fluorescent molecule 5-carboxyfluorescein diacetate acetoxymethyl ester penetrates *M. agassizii* cell membranes and is converted in the cytoplasm to the fluorescent molecule 5-carboxyfluorescein by the action of intracellular esterases. Labeled mycoplasma cells can be easily detected by flow cytometry, and cultures with fewer than 100 viable mycoplasma cells μl^{-1} can be labeled and counted in less than 1 h. Experiments using temperature-induced cell death demonstrated that only viable *M. agassizii* cells are labeled with this procedure.

A rapid and sensitive flow cytometric technique has been developed for enumerating viable *M. agassizii* cells. This technique should facilitate basic immunological, biochemical, and pharmacological studies of this important pathogen which may lead to new diagnostic and therapeutic methods.

Introduction

The desert tortoise (*Gopherus agassizii*) is found in the Mojave and Upper Sonoran deserts of Nevada, Arizona, Utah and California. Though highly adapted to life in arid environments, encroachment of civilization on desert tortoise habitat, together with periods of severe drought, have placed significant stress on these ancient reptiles. Populations of desert tortoises have declined by as much as 90% in some parts of their range, and infectious diseases are thought to be one of the causes. In particular, many desert tortoises suffer from upper respiratory tract disease (URTD), and the bacterium *Mycoplasma agassizii* has been identified as one of the causative agents (Jacobson *et al.,* 1991; Brown *et al.,*1994; Brown *et al.,* 2001).

M. agassizii is a pleiomorphic mycoplasma with an average diameter of less than 300 nm that prevents its visualization by light microscopy. Although *M. agassizii* can be cultured in the laboratory in SP4 medium at a temperature optimum of 30º C, its growth rate is extremely slow and it takes several days to visualize even slight changes in the color of the medium (color changing units). Moreover, it takes up to 8 wk for even small colonies to appear on solid agar (Wendland *et al.,* 2007; Hunter *et al.,* 2008) and these technical challenges have impeded studies of the immunology, biochemistry, and pharmacology of this important microorganism.

Flow cytometric methods for counting bacteria that provide faster results than culture methods have been developed previously (Lopez-Amoros *et al.,*

1995; Langsrud and Sundhein, 1996; Henningson *et al.,* 1998; Rattanasomboon *et al.,* 1999; Guasekera *et al.,* 2000; Holm and Jespersen, 2003). Recently flow cytometry using the DNA intercalating fluorophore SYBR Green in combination with the membrane impermeant fluorophore propidium iodide compared favorably with colony counting for determining the numbers and viability of various mycoplasma species (Assunção *et al.,*2005; Assunção *et al.,*2006a; Assunção *et al.,*2006b). We have endeavored to develop an alternative flow cytometric method for identifying and enumerating viable *M. agassizii* cells in broth culture using a single fluorophore.

Carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) is an esterified fluorogenic substrate that has been used widely for assessing esterase activity in both bacterial and mammalian cultures (Rotman and Papermeister 1966; Hoeffel *et al.,* 2003). The non-fluorescent hydrophobic molecule 5-CFDA-AM is cell permeant because of the acetoxymethyl ester. Once inside the cell the lipophilic blocking and diacetate groups are cleaved through hydrolysis by nonspecific esterases, and the resulting fluorescent 5-carboxyfluorescein (5-CF) is more polar and leaks out of the cells very slowly. We demonstrate that *M. agassizii* has a cytoplasmic esterase that can generate 5-CF from 5-CFDA-AM, and that these fluorescent molecules remain in the cell and can be used to detect and count viable *M. agassizii* by flow cytometry.

Materials and methods

Mycoplasma culture

M. agassizii was purchased from The American Type Culture Collection (ATCC No. 700616) and cultured as described (Brown *et al.,* 1994)**.** Briefly, mycoplasma cells were cultured in Spiroplasma Medium 4 (SP4) containing PPLO Broth (without crystal violet), tryptone, yeastolate yeast extract all from Becton Dickinson (Franklin Lake, NJ, USA) glucose (Fisher Scientific, Pittsburgh, PA, USA), CMRL-1066 medium (ATCC, Rockville, MD, USA), heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT, USA), and phenol red (Sigma Chemicals, St. Louis, MO, USA). Mycoplasma cells were grown in suspension cultures using an Innova 4200 incubator shaker set at 90 RPM and 30º C.

CFDA-AM staining of *M. agassizii* cells

M. agassizii cells from broth culture were centrifuged at 10,000 RPM (9279 x rcf) in an Eppendorf 5417R centrifuge at 4º C for 30 min. All subsequent washes were done with phosphate buffered saline (PBS) without Ca^{++}/Mg^{++}. Pellets were resuspended in one mL PBS, then the mycoplasma cells were stained with 1 µM CFDA-AM (Molecular Probes, Eugene, OR, USA) dissolved in dimethyl sulfoxide (DMSO) for 1 h in the dark at room temperature using an end-

over-end rocker. Cells were centrifuged and washed as before, then resuspended in 20 μl PBS. Aliquots of 5 μl were placed on glass slides under cover slips and observed through a U Plan Apochromats 60X oil immersion objective lens using an Olympus Fluo View 1000 confocal laser scanning microscope to confirm uptake of CFDA-AM and conversion to fluorescent 5-CF.

To verify that 5-CFDA-AM-labeling identified only viable cells, we measured the fluorescent intensity of *M. agassizii* cells incubated for 1 h at temperatures ranging from the optimal 30º C to the lethal 60º C using a programmable heating block. After incubation the cells were centrifuged and washed as previously described. Microorganisms were then stained with either 1 μmol ml^{-1} 5-CFDA-AM or 1 μmol ml^{-1} SYBR Green (Molecular Probes, Eugene, OR, USA) as described above. Samples were centrifuged and washed as before, pellets were resuspended in 1 ml PBS and analyzed by flow cytometry.

Flow cytometry

5-CF-stained mycoplasma cells were analyzed with an Epics XL-MCL flow cytometer (Beckman-Coulter, Miami FL, USA) equipped with an air cooled 488 nm argon-ion laser. Cells were characterized by side-angle scatter, forward-angle scatter, and green fluorescence for CF (517 nm, FL1 detector). Data were portrayed on a 4-log scale, and CF-labeled cells routinely showed a 2-log shift in fluorescence intensity. Absolute cell counts were made using Flow-Check fluorescent beads (Polysciences, Inc., Warrington, PA, USA). To avoid

coincidence each sample was tested at several dilutions to maintain a flow rate below 2000 events/second. Data were analyzed using Flo-Jo software (Tree Star, Inc., Ashland, OR, USA).

Results

Vital fluorescence labeling and imaging of *M. agassizii* cells

We first wanted to see if non-fluorescent 5-CFDA-AM molecules would be internalized by intact *M. agassizii* cells, and whether this mycoplasma possessed a cytoplasmic esterase that would hydrolyze these molecules to the fluorescent 5-CF product. Fig. 1a shows a confocal microscope image demonstrating that during a 1 h incubation 5-CFDA-AM was internalized and hydrolyzed to the fluorescent 5-CF. Similar images were obtained with incubations as short as 20 min (data not shown). These data indicate that *M. agassizii* possesses an intracellular esterase or esterases capable of hydrolyzing diacetate and acetoxymethyl esters, and demonstrate that *M. agassizii* cells can be visualized by fluorescence imaging.

Flow cytometric analysis of fluorescent-labeled *M. agassizii* cells

Next we asked whether *M. agassizii* cells labeled with 5-CFDA-AM could be detected by flow cytometry. As shown in Fig. 1b, unlabeled *M. agassizii*

showed low or no autofluorescence. These pleiomorphic cells showed some variability in side scatter, but they could be detected as discrete events despite their small size. After labeling with 5-CFDA-AM, 72% of the cells showed a greater than two log shift in fluorescence intensity (Fig1c). Some low fluorescence material remained which was most likely intact dead cells and debris. We gated on the major population of highly fluorescent cells.

Selective labeling of viable *M. agassizii* cells by 5-CFDA-AM

We used the known temperature sensitivity of mycoplasma to demonstrate that only viable *M. agassizii* cells were labeled by 5-CFDA-AM (Assunção et al., 2006a). When these organisms were grown for 1 h at temperatures ranging from a permissive 30º C to a lethal 60º C and then labeled with 5-CFDA-AM, a progressive decrease in median fluorescence intensity was observed (Fig. 2a). Almost no fluorescent signal was present in the *M. agassizii* cells grown at 60º C, perhaps because the intracellular esterases required to convert the 5-CFDA-AM to 5-CF are heat labile. Also, the *M. agassizii* cells grown at 60º C for 1 h did not exhibit growth when returned to a permissive 30º C, indicating that were not viable. A 1:1 mixture of *M. agassizii* cells grown at the permissive 30º C and the lethal 60º C was prepared, and after labeling with 5-CFDA-AM, two discrete populations of cells in equivalent numbers were observed; one with no fluorescence and the other with high fluorescence (data not shown).

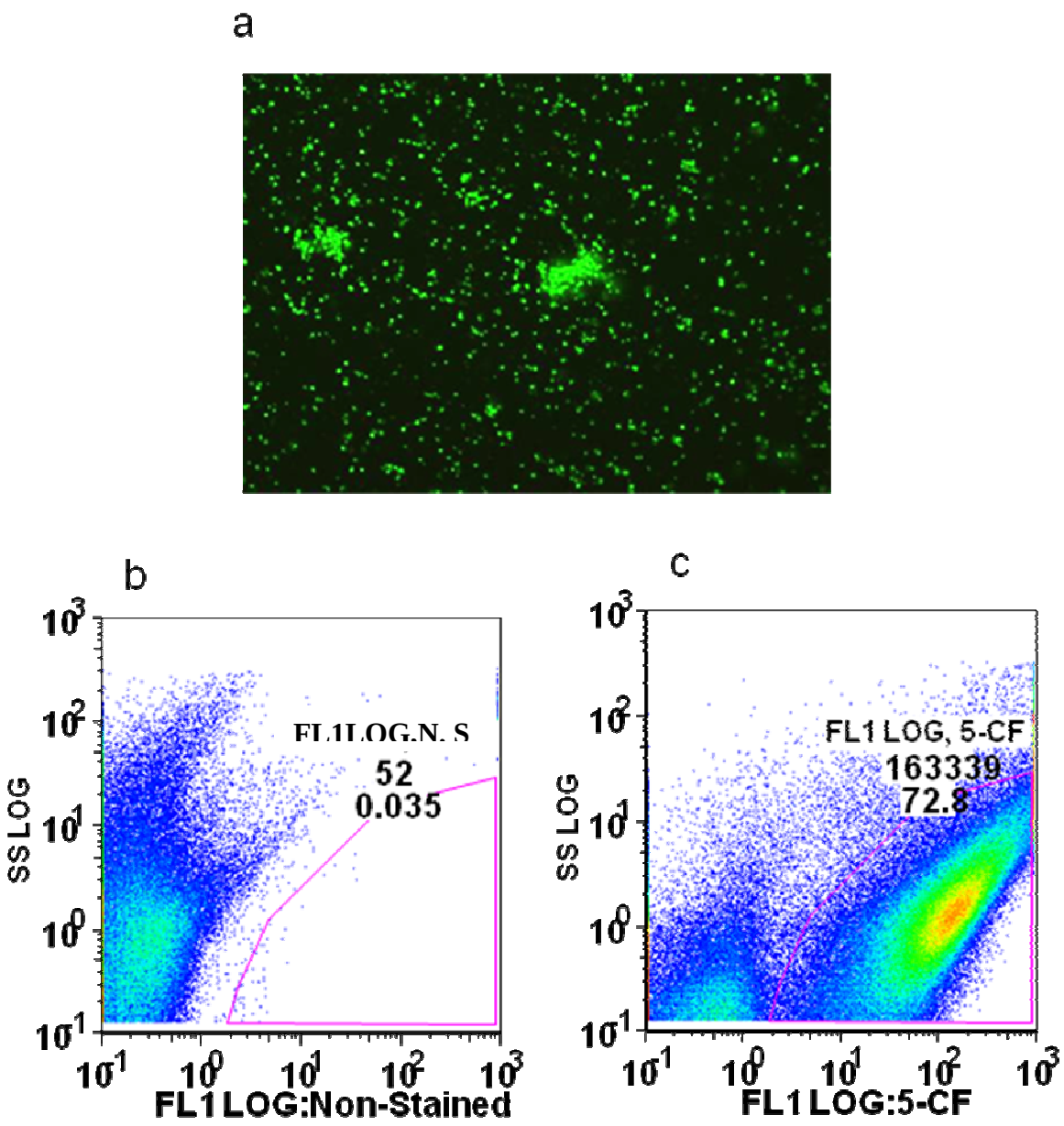

Figure 1. a. Confocal image of *M. agassizii* cells labeled *in vitro* with 5-CFDA-AM. Flow cytometric dot plots of *M. agassizii* cells before (b) and after (c) staining with 5-CFDA-AM. Unstained mycoplasma had little autofluorescence, but variable side scatter properties consistent with their pleiomorphic shape. A gate was chosen that excluded almost all unstained organisms (inset represents number of microorganisms and the % of total microorganisms in this gate). After CFDA-AM staining, 72% of the events were located within this gate; the events located outside of the gate were dead cells and cellular debris.

We then incubated *M. agassizii* cells grown for 1 h at the permissive 30º C or lethal 60º C, and then labeled with 5-CFDA-AM or SYBR Green, a DNA intercalating dye that labels both live and dead cells. While the 5-CFDA-AM-labeled cells grown at 60º C showed a decrease in fluorescence intensity to near non-stained levels (Fig. 2b), the SYBR Green-labeled cells showed no effect of temperature on fluorescence intensity (Fig. 2c). Together these data confirm that the non-fluorescent 5-CFDA-AM is internalized and converted to the fluorescent 5-CF only by viable *M. agassizii* cells.

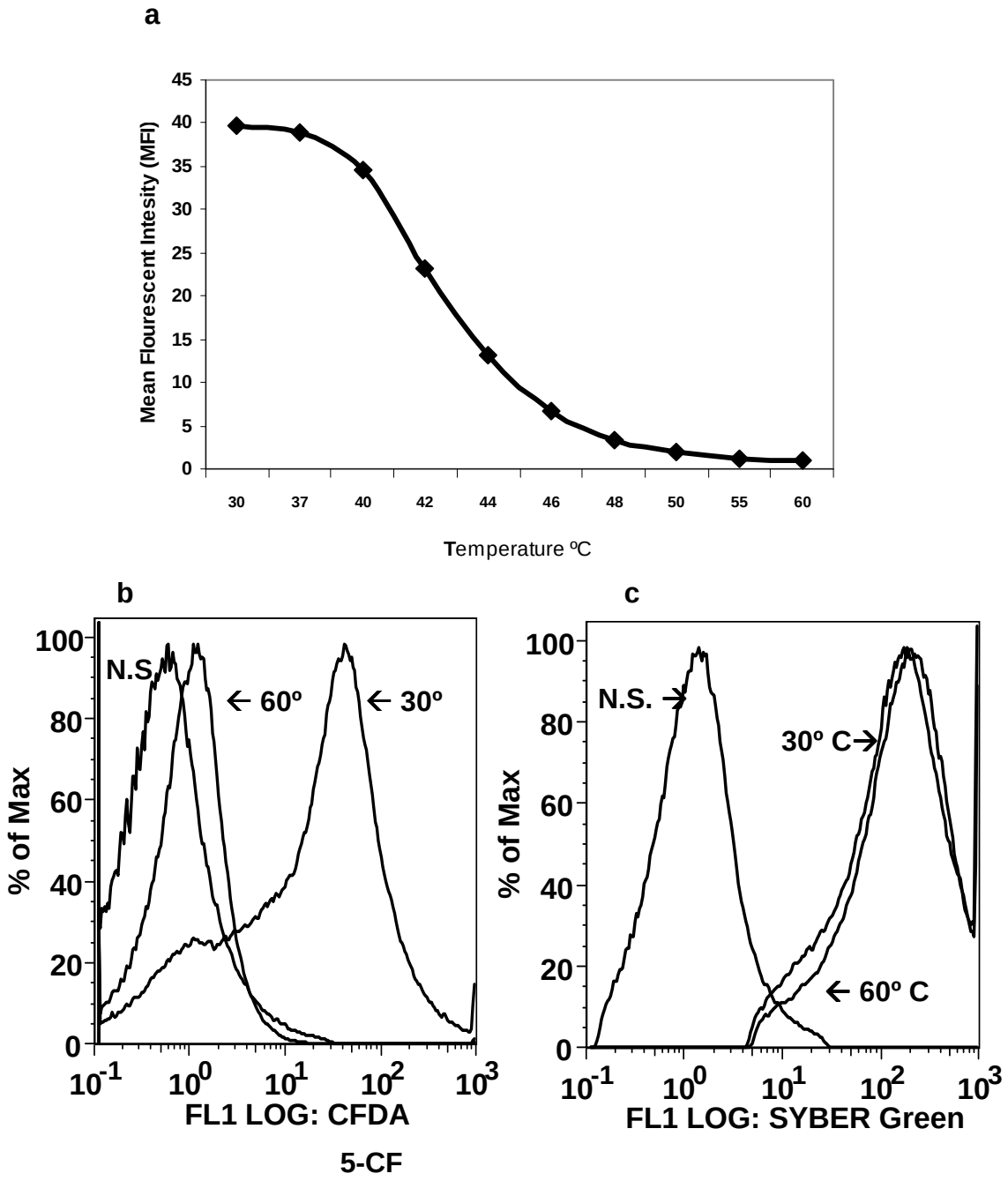

Figure 2. Effect of incubation temperature on viability of *M. agassizii* cells. (a) Cultures of 2.0 X 10^6 mycoplasma cells ml^{-1} were incubated at temperatures ranging from 30º C to 60º C for 1 h. Mean fluorescence intensity (MFI) is plotted against incubation temperature. Flow cytometric histograms showing effect of temperature on fluorescence intensity of *M. agassizii* stained with 5-CFDA-AM (b) or SYBR Green (c). Cultures were grown at 30 ℃ or 60 ℃ for 1 h then stained (N.S. = non-stained *M. agassizii*).

Assessment of *M. agassizii* growth and detection levels in broth culture

The flow cytometric method using 5-CFDA-AM staining was used to evaluate the growth of *M. agassizii* in broth culture (Fig. 3a). As expected based on previous studies *M. agassizii* growth was very slow. Although growth curves followed typical kinetics, beginning with a lag phase, followed by an exponential growth phase and finally a plateau, the doubling time was greater than 72 h. To determine the lowest number of mycoplasma cells that can be detected reliably and counted by flow cytometry, serial dilutions of 5-CFDA-AM-stained *M. agassizii* cells were analyzed by flow cytometry. We found that fewer than 100 *M. agassizii* cells ml^{-1}could be detected by this method (Fig. 3b and 3c).

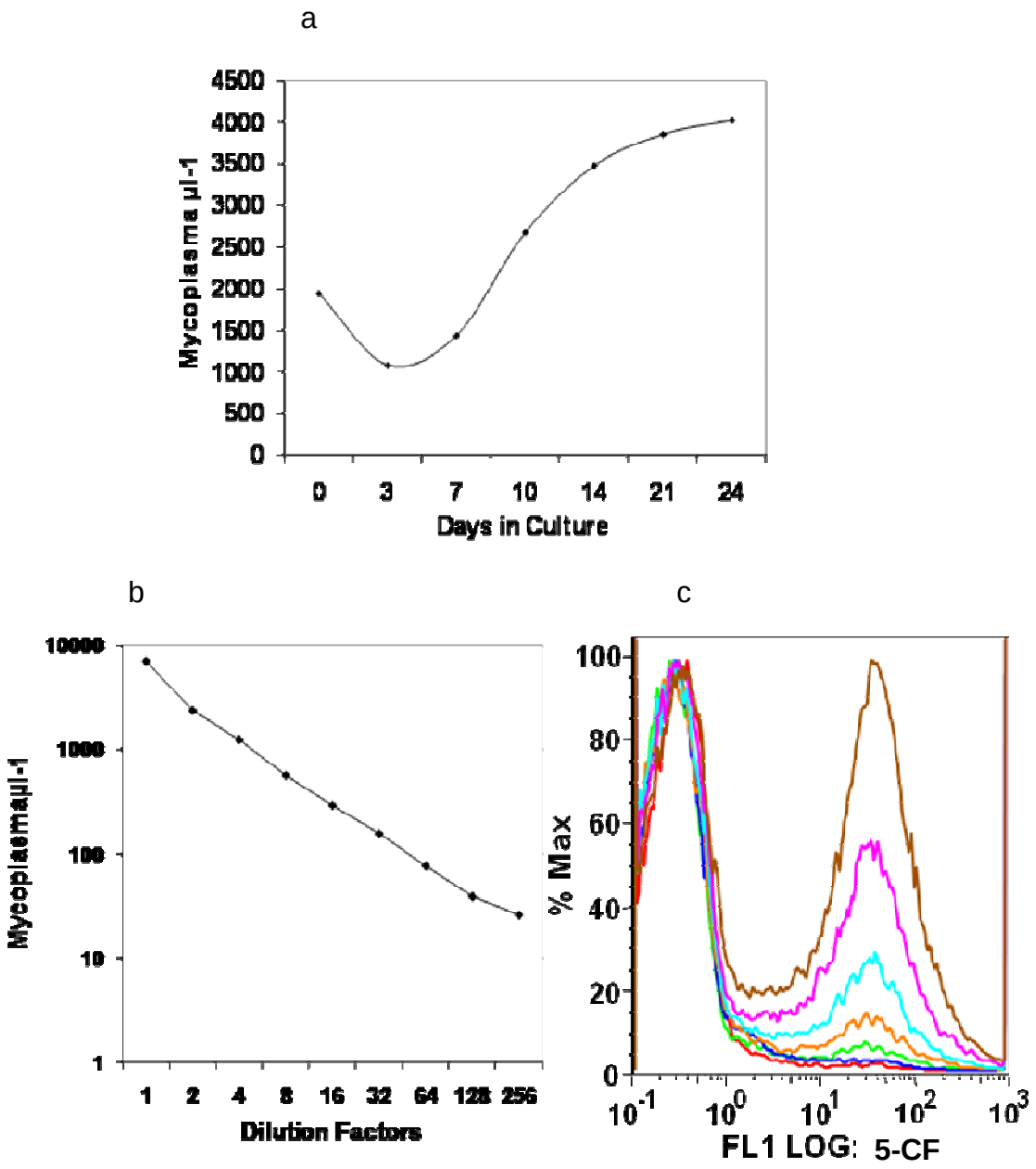

Figure 3. (a) Growth rate of *M. agassizii* in broth culture. (b) Plot of viable cell numbers stained with 5-CFDA-AM vs. dilutions demonstrating that fewer than 100 viable *M. agassizii* ml^{-1} can be detected using flow cytometry. (c) Flow cytometric histogram showing the progressive decrease in viable cell numbers at the same peak fluorescence intensity. The highest peak represents the lowest dilution shown in Figure 3b, and each lower peak in turn represents a progressively higher dilution of mycoplasma.

Discussion

Despite their small size and slow growth, Assunção *et al*., (2005) demonstrated that the mollicute *M. hypopneumoniae* could be enumerated using SYBR Green staining and flow cytometry with results comparable to colony counting. Similar findings were obtained with *M. mycoides* subsp. *mycoides* (Assunção *et al*., 2006a,b,c,d). However, because SYBR Green intercalates into the nucleic acid of both live and dead mycoplasma cells (Barber *et al*., 1971), it cannot be used to determine the number of viable microorganisms. However, in an *in vitro* study of antibacterial agents on several mycoplasma species, Assunção *et al*., (2006a, 2006b) added the cell impermeant fluorphore propidium iodide to SYBR Green and were able to distinguish live and dead mycoplasma cells by flow cytometry. This dual staining procedure worked in principle, although not all antibacterial agents (including known bacteriostatic agents) had the same effect on membrane permeability.

In contrast to many mycoplasma species, *M. agassizii* grows very poorly in standard SP4 medium (Wendland *et al*., 2007; Hunter *et al*., 2008). Indeed, the growth rate is too slow to accurately use color changing units in broth culture, and it takes six to eight weeks to observe even small colonies on agar medium (Fig S1). We were unable to find quantitative relationships when using colony counts (data not shown). Moreover, for many experimental purposes, it is desirable to know the numbers of viable microorganisms at the time research studies are performed. The purpose of this study was to show that a single

fluorescent label can be used to rapidly enumerate viable *M. agassizii* by flow cytometry.

The internalization of non-polar, non-fluorescent molecules and their conversion by the action of intracellular enzymes (such as esterases) to polar, fluorescent compounds that are retained within living cells has been shown to be a reliable measure of mammalian cell viability (Prosperi *et al*.,1986). Here we show that non-fluorescent 5-CFDA-AM molecules, once internalized by viable *M. agassizii*, are converted into fluorescent 5-CF molecules through esterase hydrolysis and retained within the cells (Fig.S2). By culturing *M. agassizii* organisms at a permissive 30º C and a lethal 60º C we were able to show that only viable mycoplasma are stained with 5-CF. In addition this method allowed for the enumeration of fewer than 100 viable microbes ml^{-1}, which compares to the detection limit reported for various mycoplasma species in goat milk (Assunção *et al*., 2007). The ability to detect and enumerate viable *M. agassizii* in broth cultures by 5-CFDA-AM staining allows the researcher to perform a variety of experiments with these organisms in the laboratory which were previously not possible using conventional culture methods.

References

Assunção, P., Diaz, R., Comas, J., Ruiz de Galarreta, C.M., Llamazares, O.G. and Poveda, J.B. (2005). Evaluation of *Mycoplasma hyopneumoniae* growth by flow cytometry. *J. Appl. Microbiol.* **98**, 1047–1054.

Assunção, P., Antunes, N.T., Rosales, R.S., de la Fe, C., Poveda, C., Poveda, J.B and Davey, H.M. (2006a). Flow cytometric method for the assessment of the minimal inhibitory concentrations of antibacterial agents to *Mycoplasma agalactiae*. *Cytometry A* **69**, 1071-1076.

Assunção, P., Antunes, N.T., Rosales, R.S., Poveda, C., de la Fe, C., Poveda, J.B and Davey, H.M. (2006b). Application of flow cytometry for the determination of minimal inhibitory concentration of several antibacterial agents on *Mycoplasma hyopneumoniae*. *J. Appl. Microbiol.* **102**, 1132-1137.

Assunção, P., Antunes, N.T., Rosales, R.S., Poveda, C., Poveda, J.B and Davey, H.M. (2006c). Flow cytometric determination of the effects of *antibacterial agents on Mycoplasma agalactiae, Mycoplasma putrefaciens, Mycoplasma capricolum* subsp. *capricolum*, and *Mycoplasma mycoides* subsp. *mycoides* large colony type. *Antimicrob. Agents Chemother.* **50**, 2845-2849.

Assunção, P., de la Fe, C., Antunes, N.T., Rosales, R.S., Ruiz de Galarra, C.M. and Poveda, J.B. (2006d). Use of flow cytometry for enumeration of *Mycoplasma mycoides* subsp. Mycoides large-colony type in broth medium *J. Appl. Microbiol.* **100,** 878-884.

Assunção, P., Davey, H.M., Rosales, R.S., Antunes, N.T., de la Fe, C. ,Ramirez, A.S., de Galarreta, C. M. and Poveda, J.B. (2007). Detection of mycoplasma in goat milk by flow cytometry. *Cytometry Part A* **71**, 1034-1038.

Barber, T.L and Fabricat, J. (1971). Identification of *Mycoplasmatales*: characterization procedures. *J. Appl. Microbiol.* **21**, 600-605.

Brown, M.B., Schumacher, I.M., Klein,P.A., Harris, R.K., Correll, T.and Jacobson, E. (1994). *Mycoplasma agassizii* causes upper respiratory tract disease in desert tortoise. *Infect. Immun.* **62**, 4580-4586.

Brown, M.B., Brown, D.R., Klein, P.A., McLaughlin, G.S, Schumacher, I.M., Jacobson, E.R., Adams, H.P. and Tully, J.G. (2001). *Mycoplasma agassizii* sp. nov., isolated from the upper respiratory tract of the desert tortoise (*Gopherus agassizii*) and the gopher tortoise (*Gopherus polyphemus*). *Int. J. Syst. Evol. Microbiol.* **1,** 413-418.

Gunasekera, T.S., Attfield, P.V. and Veal, D.A. (2000). A flow cytometry method for rapid detection and enumeration of total bacteria in milk. *Appl. Environ. Microbiol.* **66**, 1228-1232.

Henningson, E.W., Corcova, Z., Sandstrom, G. and Forsman, M. (1998). Flow cytometric assessment of the survival ratio of *Francisella tularensis* in aerobiological samples. *FEMS Microbiol. Ecol.* **25**, 241-249.

Hoefel, D., Warwick, L.G., Monis, P.T., Andrews, S and Saint, C.P. (2003). A comparative study of carboxyfluorescein diacetate and carboxyfluorescein diacetate succinimidyl ester as indicators of bacterial activity. *J. Microbiol. Meth.* **52**, 379-388.

Holm, C. and Jespersen, l. (2003). A flow-cytometric gram-staining technique for milk-associated bacteria. *Appl. Environ. Microbiol.* **69**, 2857-2863.

Hunter, K.W. Jr., Dupré, S.A., Sharp, T., Sandmeier, F.C and Tracy, C.R. (2008). Western blot can distinguish natural and acquired antibodies to *Mycoplasma agassizii* in the desert tortoise (*Gopherus agassizii*). *J. Microbiol. Meth.* **75**, 464-471.

Jacobson, E.R., Gaskin, J.M., Brown, M.B., Harris R.K., Gardiner C.H., LaPointe J.L., Adams, H.P. and Reggiardo C. (1991). Chronic upper respiratory tract disease of free-ranging desert tortoises (*Xerobates agassizii*). *J. Wildl. Dis.* **27,** 296-316.

Langsrud, S. and Sundheim, G. (1996). Flow cytometry for rapid assessment of viability after exposure to a quaternary ammonium compound. *J. Appl. Bacteriol.* **81**, 411-418.

Lopez-Amoros, R., Comas, J. and Vivas-Rego, J. (1995). Flow cytometric assessment of Escherichia coli and *Salmonella typhimurium* starvation-survival in seawater using rhodamine 123, propidium iodide and oxonol. *Appl. Environ. Microbiol.* **61**, 2521-2526.

Prosperi, E., Croce, A.C., Bottiroli, G. and Supino, R. (1986). Flow cytometric analysis of membrane permeability properties influencing intercellular accumulation and efflux of fluorescein. *Cytometry* **7**, 70-75.

Rattanasomboon, N., Bellara, S.R., Harding, C.L., Fryer, P.J., Thomas, C.R., Al-Rubeai, M. and McFarlane, C.M. (1999). Growth and enumeration of the meta spoilage bacterium *Brochothrix thermosphacta*. *Inte.r J. Food. Microbiol.* **51**, 145-158.

Rotman, B., and Papermaster, B.W. (1966). Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. *Proc. Nat. Acad. Sci.* USA **55**, 134-141.

Wendland, L.D., Zacher, L.A., Klein, P.A., Brown, D.R., Demcovitz, D., Littell, R. and Brown, M.B. (2007). Improved enzyme-linked immunosorbent assay to reveal *Mycoplasma agassizii* exposure: a valuable tool in the management of environmentally sensitive tortoise populations. *Clin. Vaccine. Immunol.* **14,** 1190-1195.

Chapter Three

Preparation and characterization of rabbit polyclonal antibodies to the major immunoglobulin isotypes of the desert tortoise (*Gopherus agassizii*)

Abstract

To facilitate seroepidemiologic studies of infectious diseases in the threatened desert tortoise (*Gopherus agassizii*) isotype-specific antibody reagents are needed. Our goal was to prepare isotype-specific rabbit polyclonal antibodies to desert tortoise IgM and IgY.

A 50% saturated ammonium sulfate precipitate of desert tortoise serum was fractionated by Sephacryl A-300 gel filtration chromatography and IgM-rich and IgY-rich fractions were collected. SDS-PAGE confirmed the presumed molecular weights of the heavy chains of tortoise IgM and IgY to be 71 kD and 68 kD respectively. Rabbits immunized with these separate fractions produced high titer sera that cross-reacted slightly with the opposite heavy chain and strongly with the conserved 22 kD light chain. To make heavy chain isotype-specific reagents, the Ig fraction of each antiserum was cross-absorbed using polystyrene microspheres coated with the opposite isotype.

Enzyme-linked Immunosorbent Assay (ELISA) and Western blots confirmed the heavy chain specificity of the cross-absorbed antibodies. These high avidity rabbit polyclonal antibody reagents were used in ELISA and Western blots to document the primary and secondary isotype-specific antibody responses to *Mycoplasma agassizii* infection and ovalbumin immunizations in desert tortoises.

These new rabbit polyclonal antibodies will allow more detailed studies of the humoral immune response of the desert tortoise.

Introduction

Populations of the desert tortoise (*Gopherus agassizii*) in the Southwestern United States have declined dangerously over the last three decades (U.S. Fish and Wildlife Service, 1990; U.S. Fish and Wildlife Service, 2011). Upper respiratory tract disease (URTD) is considered to be a cause of morbidity and mortality in wild populations, and two species of mycoplasma (*Mycoplasma agassizii* and *M. testudineum*) are known to be causative agents (Brown *et al*., 1994; Brown *et al*., 2004). In addition, *Pasteurella testudinis* (Snipes and Fowler, 1980) and a Herpes virus (Pettan-Brewer *et al*., 1996; Johnson *et al*., 2005; Jacobson, 2007) may also be associated with the disease. Historically, serodiagnostic studies of mycoplasmosis in the desert tortoise have relied on reagents that do not distinguish among the major immunoglobulin (Ig) isotypes or classes (Brown *et al*., 1994; Hunter *et al*., 2009). Reptiles like the desert tortoise are thought to have two major Ig isotypes (IgM and IgY), and a truncated version of IgY called IgY (ΔFc) that is missing the two carboxy terminal heavy chain domains of IgY (Benedict and Pollard, 1972; Leslie and Clem, 1972). Most studies of mycoplasmosis have used a mouse monoclonal antibody reagent that recognizes the tortoise light chain that is thought to be conserved among the heavy chain isotypes (Brown *et al*., 1994). A rabbit polyclonal antibody reagent has also been developed that recognizes the light chain and the heavy chains of all three isotypes of desert tortoise Ig (Hunter *et al*., 2009).

In mammalian immune responses, IgM is the earliest isotype produced in response to antigenic stimulation, followed by a switch to different isotypes (Flajnik *et al*., 2003). There is evidence that isotype switching also occurs in reptiles (Turchin and Hsu, 1996), and is presumed to occur in the desert tortoise. To facilitate further seroepidemiologic studies of infectious diseases in the desert tortoise we have developed isotype-specific rabbit polyclonal antibody reagents to the heavy chains of IgM and IgY. We demonstrate their use following humoral immune responses first in desert tortoises given intradermal immunizations with ovalbumin (OVA), and second, after intranasal infection with the URTD pathogen *M. agassizii*.

Materials and methods

Animals, immunizations/infections, and blood collection

Male and female desert tortoises (*Gopherus agassizii)* were maintained in the University of Nevada, Reno vivarium in controlled light and temperature conditions according to established husbandry procedures (Hunter *et al*., 2009). In our first study three tortoises were immunized intradermally with 1 mg ovalbumin (Sigma Chemicals, St. Louis, MO) emulsified in Ribi adjuvant (Sigma-Aldrich, St. Louis, MO), and two control animals were immunized with PBS . These initial immunizations were followed by a booster immunization at day 60. In our second experiment, six tortoises were inoculated intranasally with 3.8 x 10^8 cells of the SP6 strain of M. agassizii (Brown *et al*., 1994) in 0.5 mL of sterile

PBS. Flow cytometry was used to determine the viable mycoplasma inoculum (Mohammadpour *et al*., 2010). Of the tortoises inoculated with *M. agassizii*, only one of the six (L26) seroconverted during the subsequent three month observation period. This tortoise was used to demonstrate the isotype-specific antibody response following infection. Tortoise blood samples (1.5 ml) were obtained from the subcarapacial sinus (Hernandez *et al*., 2002). New Zealand white rabbits (Pacific Immunology, San Diego, CA) were immunized with partially purified tortoise IgM and IgY emulsified in complete Freund's adjuvant, followed at day 30 by a single booster immunization with the same material emulsified in incomplete Freund's adjuvant. After clotting, rabbit sera were separated by centrifugation and stored at -80℃. All procedures were approved by the University of Nevada, Reno Institutional Animal Care and Use Committee, and desert tortoises were sampled under permits issued by the U.S. Fish and Wildlife Service and the Nevada Department of Wildlife.

Preparation of tortoise Ig and enrichment for IgM and IgY

Pooled serum collected as described above served as the source of tortoise Ig. As a preliminary step in isolating tortoise Ig, saturated ammonium sulfate was added to the pooled serum to achieve a 25% final concentration (v/v). After several hours of stirring at room temperature, non-Ig aggregates were removed by centrifugation at 3000 x g for 10 min. The supernatant was then adjusted to a final 50% ammonium sulfate concentration and stirred overnight at 4ºC. The precipitated tortoise Ig was collected by centrifugation at 3000 x g for 10

min, then the precipitate was redissolved in phosphate buffered saline (PBS, pH 7.2) and dialyzed at 4ºC against 4 L of the same buffer. Protein content of the dialyzed Ig was determined by the bicinchoninic acid protein assay (BCA) (ThermoScientific, Rockford, IL). A 20 mg aliquot of Ig in a 1 mL volume was fractionated on a Sephacryl A-300 column (1.5 x 50 cm) (Bio-Rad, Hercules, CA) using a flow rate of 0.5 ml min^{-1}. One ml fractions were collected and the protein content determined by spectrophotometry (O.D. 280 nm). The void volume peak with intact pentavalent IgM (presumed molecular weight >950,000 Da), and a second IgY-rich peak were separately pooled, concentrated, and examined by SDS-PAGE. Briefly, 10 µg of each pooled fraction was denatured in sample loading buffer (containing 12.5 mM Tris-HCl, 0.5% sodium dodecyl sulfate, 2.5% glycerol, 3.125 mM EDTA, and 0.5% β- mercaptoethanol) in boiling water for 20 min. Proteins were initially separated on a 4-20% polyacrylamide gel (Bio-Rad, Hercules, CA) to identify and confirm molecular size. Gels were run under a constant 20 mA current until the bromophenol dye indicator reached the bottom. For Western blot analysis, proteins were separated on 10% Tris HCl acrylamide gels and were blotted onto a 0.45 µm nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 5% non-fat dried milk in Tris buffered saline containing 0.05% Tween-20 (TBS-T, Sigma Chemical, St Louis, MO) for 2 h and washed three times with TBS-T. Tortoise plasma diluted 1:100 in TBS-T plus 5% milk was added onto the membrane and incubated overnight at 4ºC. Membranes were washed three times with TBS-T followed by incubation with either rabbit anti-tortoise IgM (RaTIgM) or rabbit anti-tortoise IgY (RaTIgY) at

1:20,000 dilutions for 2 h at room temperature. After washing as before, horseradish peroxidase conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA) was added at a 1:5,000 dilution for 1 h at room temperature. Following a final three washes, blots were developed using metal enhanced 3, 3'-diaminobenzidine (DAB, Pierce Rockford, IL). Color development was stopped by washing the membrane with water. Dried blots were photographed using a Gel-doc (Bio-Rad, Hercules, CA

Preparation of isotype-specific rabbit antibodies by cross-absorption

Polystyrene microspheres (5 µm diameter) were obtained from Bangs Laboratories (Fishers, IN). The microspheres (1 mg) were placed in small tubes and washed with PBS several times. They were mixed for 24 h at 4ºC with 100 µg ml^{-1} of partially purified tortoise IgM or tortoise IgY in PBS. The microspheres were washed four times with PBS, and then blocked with 5% non-fat dry milk for 2 h at room temperature with mixing. After an additional four washes with PBS containing 0.05% Tween 20 (PBS-T) the tortoise IgM and IgY-coated microspheres were used for cross-absorption. RaTIgM and RaTIgY were each diluted with PBS to a stock protein concentration of 100 µg ml^{-1}. An aliquot of 0.5 ml of RaTIgM stock solution was diluted 1:100 in PBS and added to 0.5 mg of tortoise IgY-coated microspheres, and the reciprocal cross-absorption was done with RaTIgY and tortoise IgM-coated microspheres. The absorption was allowed to proceed for 2 h at 4ºC with constant mixing, followed by centrifugation at 6000

x g in a micro centrifuge (Beckman Coulter, Miami, FL). This cross-absorption was repeated three times, followed by three PBS-T washes.

ELISA and Western blots

Enzyme-linked Immunosorbent Assay (ELISA) and Western blots for M. agassizii were performed as previously described (Hunter *et al*., 2009) except for the use of the isotype-specific reagents. For the mycoplasma ELISA, RaTIgM and RaTIgY stock reagents were used at 1:10,000 dilutions, while 1:5,000 dilutions were used for Western blots. In the ELISA procedure for measuring the isotype-specific antibody response to OVA, polystyrene micro titer plates were coated overnight with a 10 µg ml^{-1} solution of OVA in PBS. After washing three times with PBS, the plates were blocked with 5% non-fat dry milk in PBS for 2 h at 4ºC. Following another three washes with PBS-T, various dilutions of plasma from OVA immunized tortoises were added to the plates. After 2 h incubation at room temperature, the plates were washed three times with PBS-T. Either RaTIgM or RaTIgY in PBS-T at a 1:10,000 dilution was added to wells and incubated at room temperature for 1 h. Wells were washed as before and 50 µl of a secondary goat anti-rabbit horseradish peroxidase-conjugated antibody was added at 1:5,000 and incubated for one hr at room temperature. Plates were washed three times in PBS-T and developed by adding 50 µl of 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate (KPL, Gaithersburg, ML) solution for 30 min in the dark at room temperature. The reaction was stopped by

adding 50 µl of 0.5 N hydrochloric acid (HCl) solution and the optical density was measured at 450 nm using a Spectromax microplate reader (Molecular Devices, Sunnyvale, CA).

Analysis of ELISA data

Sample titers were determined as described (Hunter et al., 2009) by plotting the mean of the optical density (450nm) versus log plasma dilutions. Logarithmic curve fit was determined for the linear portions of each line [y = a ln(x) + b]. An optical density in the mid portion of the linear curves (e.g., 1.0 optical density units) was used to determine the x value for each serum dilution. The titer was defined as the reciprocal of the x value corresponding to the selected y value.

Results

Isolation and characterization of IgM and IgY-rich fractions of tortoise Ig

Previous work with other chelonians (Christo and Botyo, 1988) suggested that the IgM molecule is a pentamer with a molecular weight >900 kD. We elected to run tortoise Ig over a Sephacryl-A300 column, and to collect the high molecular weight IgM in the void volume. The lower molecular weights of IgY and IgY(ΔFc) allowed these molecules to traverse the gel beads and to separate as a separate IgY-rich peak. Fig. 1a shows the chromatographic profile of a representative sample separation. Each pooled fraction (as indicated on the chromatogram) was tested by SDS-PAGE to identify the banding patterns. As shown in Fig. 1b, the IgM fraction had a prominent heavy chain band at 72 kD, and the IgY-rich fraction had a prominent band at 68 kD. Both fractions also showed prominent bands at 22 kD, representing the light chain. The IgY-rich fraction also had a band at approximately 38 kD which probably represented the IgY (ΔFc) heavy chain (Benedict and Pollard, 1972; Leslie and Clem, 1972) There were several other contaminating bands as expected from the use of pooled tortoise Ig as the starting material. The IgM-rich and IgY-rich material was used to immunize rabbits as described above.

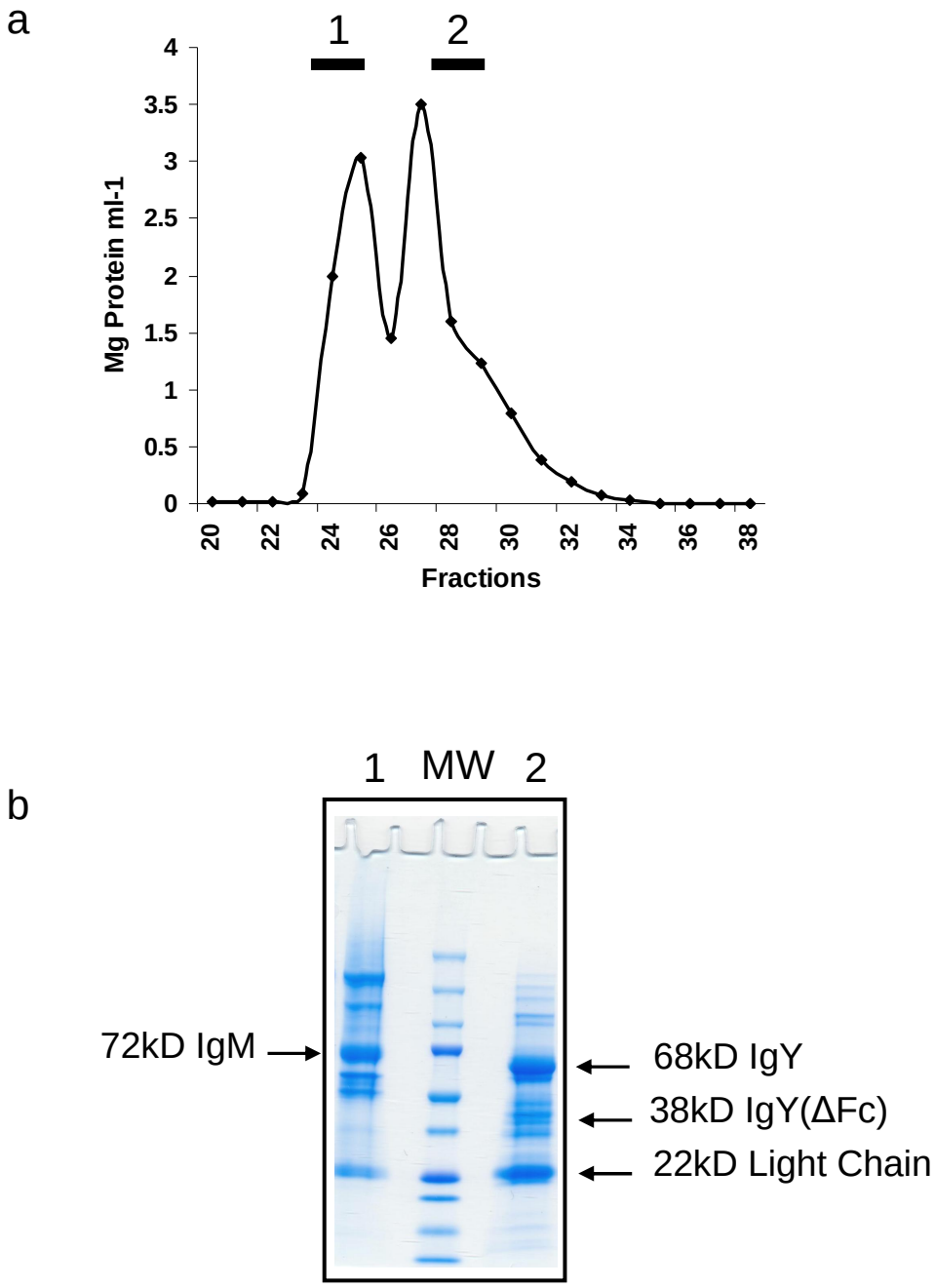

Figure 1 (a) Fractionation of tortoise Ig on a Sephacryl-A300 column. Bars indicated pooled fractions 1 and 2. (b) Coomassie stain of a Tris HCl acrylamide gradient gel (4-20%) used in the SDS-PAGE analysis of IgM-rich (lane 1) and IgY-rich (lane 2) fractions. MW = molecular weight markers.

Testing of tortoise IgM and IgY-specific antibodies prepared by cross-absorption

When initially tested by Western blot, both rabbit antisera to tortoise IgM and IgY showed weak cross-reactivity with the heavy and light chains of the opposite isotype (Fig. 2a and 2b). There was also immunological reactivity against several of the contaminating proteins. However, after multiple cross-absorptions against Ig of the opposite isotype, isotype-specific RaTIgM and RaTIgY reagents were generated (Fig. 2c and 2d).

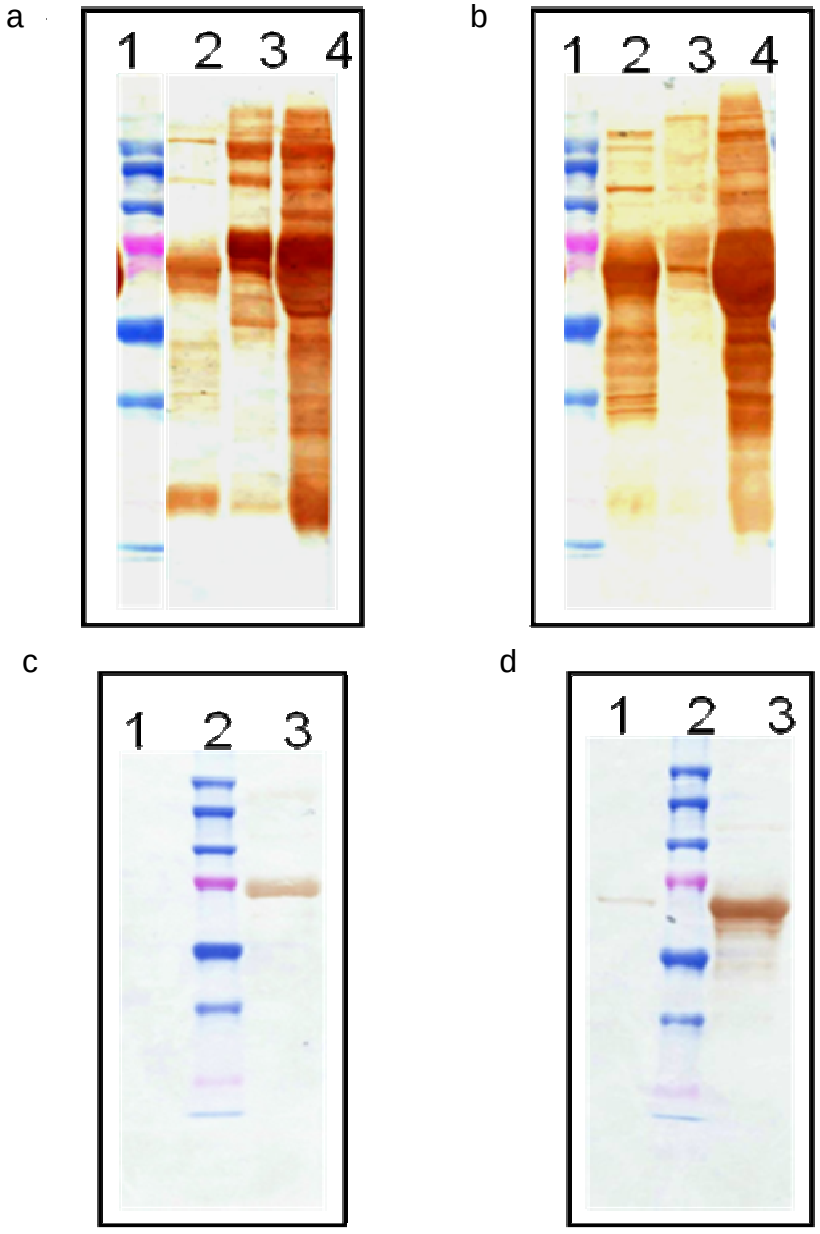

Figure 2 Western blots probed with either RaTIgM or RaTIgY before and after cross-absorption. (a) Lanes 2-4 represent the IgY-rich, IgM-rich, and total Ig fractions probed with RaTIgM before cross-absorption. (b) Lanes 2-4 represent the IgY-rich, IgM-rich, and total Ig fractions probed with RaTIgY before cross-absorption. (c) Lanes 1 and 3 represent the IgY-rich and IgM-rich fractions probed with RaTIgM after cross-absorption. (d) Lanes 1 and 3 represent the IgM-rich and IgY-rich fractions probed with RaTIgY after cross-absorption. MW markers are indicated on each gel.

Demonstration of the isotype-specific primary and secondary antibody responses of desert tortoises following immunization with OVA

Two of the three desert tortoises immunized with OVA showed an initial IgM specific response. All three tortoises demonstrated slow developing IgY responses that may have been augmented by the booster immunization at 60 days. The IgY titers peaked at above 1:35,000 after day 100 in all three tortoises, nearly two months after the final booster immunization.

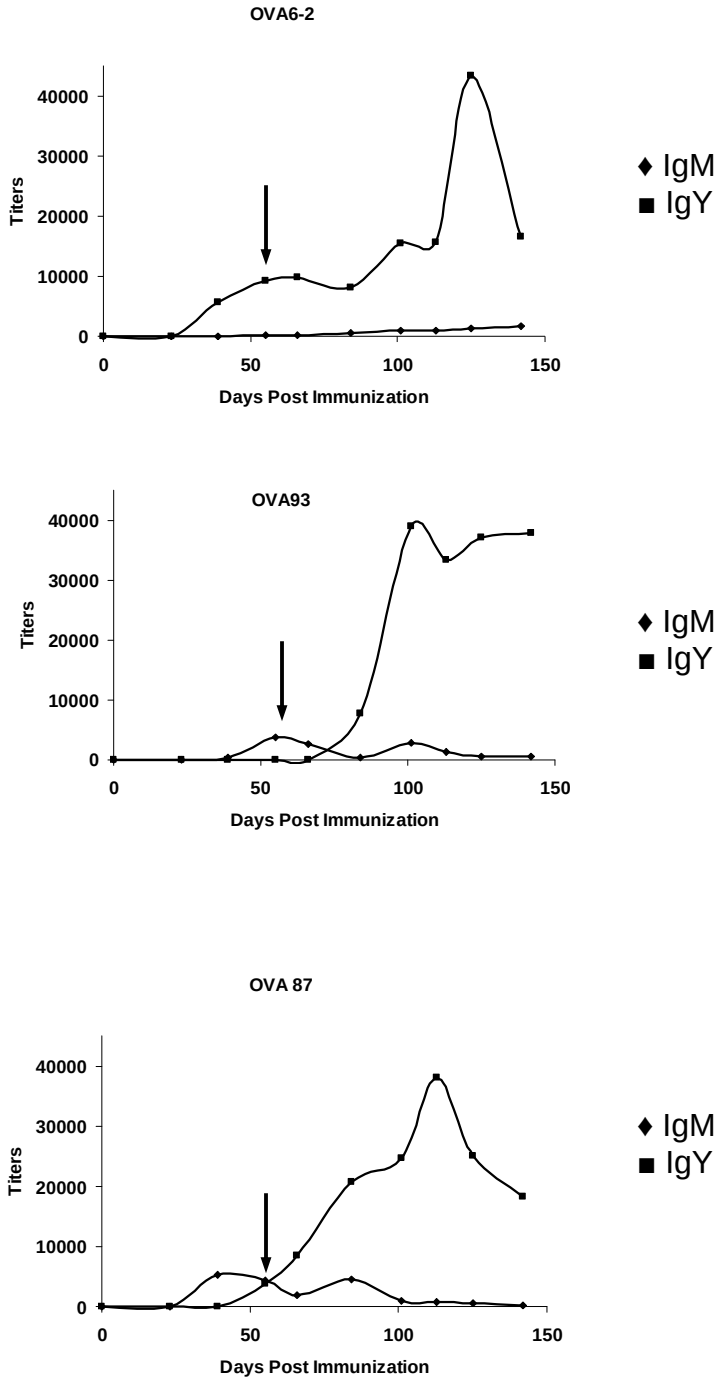

Figure 3 IgM and IgY-specific responses of three OVA-immunized desert tortoises. Secondary immunizations were given on day 60 (black arrow). ELISA titers were calculated as described in the Materials and Methods section.

Demonstration of isotype-specific antibody response of a desert tortoise following intranasal infection with *Mycoplasma agassizii*

Six desert tortoises were given intranasal inoculations of 3.5×10^8 viable mycoplasma cells, but only one of the tortoises initially showed seroconversion. It should also be noted that no outward clinical signs or symptoms of URTD were observed in any of the tortoises. Tortoise L26 demonstrated a slowly rising early IgM response (Fig. 4a), followed by a more rapid increase in IgY beginning at day 50. The IgY levels increased steadily to a peak ELISA titer of approximately 1:25,000 at around 6 mo post-infection, then the titer began to decrease over the next month. An ELISA titer over 1:15,000 was still observed eight months post-infection (data not shown).

Isotype-specific Western blots were also performed on the serum samples from tortoise L26 (Fig. 4b and 4c). Pre-infection serum showed several bands developed by both the IgM and IgY reagents consistent with our previous identification of natural antibodies to *M. agassizii* in non-infected desert tortoises (Hunter *et al*., 2009). As the infection progressed, both the IgM and IgY-specific responses expanded to identify more mycoplasma antigens, though the IgY response was much more robust by day 64.

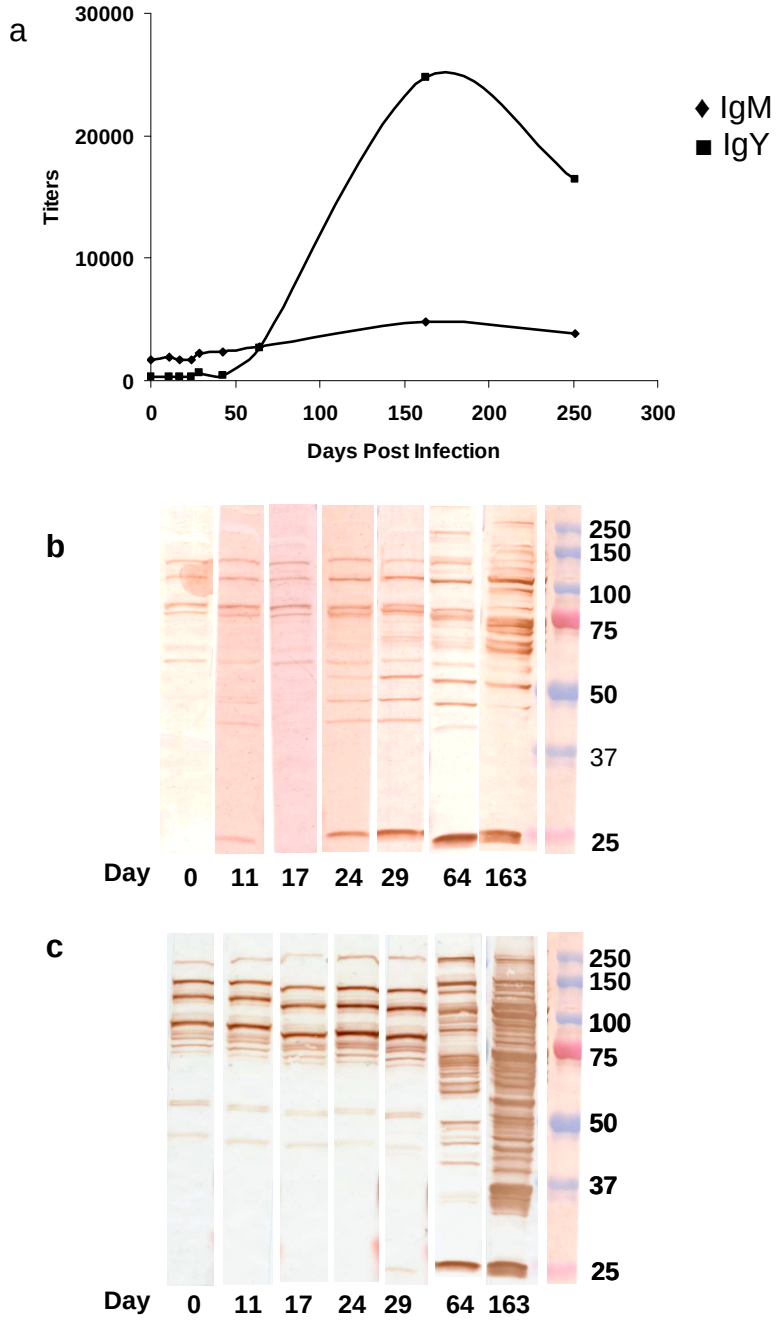

Figure 4. IgM and IgY antibody responses of desert tortoise L26 following intranasal infection with *M. agassizii*. (a) Isotype-specific responses measured by ELISA. (b) IgM antibody response measured by Western blot (c). IgY antibody response measured by Western blot.

Discussion

In the present study we have used cross-absorption to generate IgM and IgY heavy chain specific rabbit polyclonal antibody reagents to facilitate the study of humoral immune responses in the desert tortoise. Using a combination of SDS-PAGE and Western blotting, we demonstrated the heavy chain specificity of the reagents, and also showed that the apparent molecular weights of the IgM and IgY heavy chains were 72 kD and 68 kD respectively. This is consistent with the early work of Herbst and Klein (1995) who found similar molecular weights for the IgM and IgY heavy chains of the green sea turtle *Chelonia mydas*.

To demonstrate the utility of the isotype-specific reagents, we performed two small studies with captive desert tortoises. First, three desert tortoises were immunized with OVA in adjuvant, followed by booster immunizations at day 60. Blood samples were obtained periodically and analyzed by ELISA using the isotype-specific reagents. Two of the three immunized tortoises made an early IgM antibody response to OVA (Fig. 3b and 3c). The IgY response to OVA was detected in all three tortoises by day 50, and after a booster immunization at day 60 the levels of these antibodies continued to rise significantly over the next two months. This pattern of an early, but modest, IgM response followed by a more robust IgY response is similar to the pattern seen in mammals, but at a very different time scale. The data are consistent with the notion that the desert tortoise undergoes isotype switching during an antibody response, though this will require molecular studies for verification.

A second study was performed to examine the isotype-specific immune response to *M. agassizii*, a desert tortoise pathogen (Schumacher, *et al*., 1993; Brown *et al*., 1994). In this study, six tortoises were given intranasal inoculations of viable *M. agassizii* cells. Pre-infection blood samples from all six tortoises had IgM and IgY natural antibodies to *M. agassizii* antigens, consistent with our previous observation (Hunter *et al*., 2009). It is interesting to note, however, that the natural antibody banding pattern developed with the IgM and IgY-specific reagents was different. Blood samples were obtained periodically and analyzed by both ELISA and Western blot using the isotype-specific reagents. Only one of the six tortoises (L26) serconverted following intranasal inoculation. The isotype-specific antibody response to *M. agassizii* was followed in this tortoise over the course of the next three months. The initial antibody response observed in tortoise L26 was predominantly of the IgM class. IgM ELISA titers increased to a peak at day 150, and then decreased over the next month. The changing antigen specificity of this IgM response was revealed by Western blot as the addition of many new bands to the initial natural antibody banding pattern. In contrast, the IgY response was delayed by nearly a month, but then the antibody levels increased markedly and peaked at a titer of 1:25,000 on day 150 post-infection. Western blot revealed an increasing number of bands over time, culminating in a very robust pattern with more than 45 identifiable bands by day 163 post-infection. Although only one of the tortoises (L26) seroconverted over the course of the 64 day experiment, the remaining five tortoises did seroconvert at an

unknown later date, all six tortoises were reanalyzed three years post infection and showed seroconversion (Fig S3).

This study demonstrated the usefulness of our isotype-specific rabbit anti-tortoise IgM and IgY reagents in following the natural course of an infection in the desert tortoise. The changing pattern of antibody reactivity with antigens of *M. agassizii* revealed by Western blot, and the isotype-specificity of this response, provide another tool to assess the seroepidemiology of infectious diseases like mycoplasmosis in the desert tortoise.

References

Benedict, A.A. and Pollard, L.W. (1972) Three classes of immunoglobulins found in the sea turtle, *Chelonia mydas*. *Folia Microbiol.* **17**, 75–78.

Brown, D.R., Merritt, J.L., Jacobson, E.R., Klein, P.A., Tully, J.G., and Brown, M.B. (2004) *Mycoplasma testudineum* sp. nov., from a desert tortoise (*Gopherus agassizii*) with upper respiratory tract diseases. *Inter. J. Syst. Evol. Microbiol.* **54**, 1527-1529.

Brown, M.B., Schumacher, I.M., Klein, P.A., Harris, K., Correll, T. and Jacobson, E.R. (1994) *Mycoplasma agassizii* causes upper respiratory tract disease in the desert tortoise. *Infect. Immun.* **62**, 4580–4586.

Christo, L.A. and Botyo, A.B. (1988) Isolation and Partial Characterization Of IgM-Like Immunoglobulins From the serum of carp (*Cyprinus carpio* L), frog (*Rana ridibunda* Pall) and Tortoise (*Testudo graeca* Pall). *Comp. Biochem. Physiol. Part D: Comp. Biochem.* **89**, 737-741.

Flajnik, M.F., Miller, K. and du Pasquier, L. (2003) In: Paul, W.E. (Ed.), Fundamental Immunology. Lippincott Williams &Wilkins, Philadelphia, pp. 519–570.

Herbst, L.H. and Klein, P.H. (1995) Monoclonal antibodies for the measurement of class-specific antibody responses in the green turtle, *Chelonia mydas*. *Vet. Immunol. Immunopathol.* **46**, 317-335.

Hernandez-Divers, S.M., Hernandez-Divers, S.J. and Wwyneken, J. (2002) Angiographic, anatomic and clinical technique descriptions of a subcarapacial venipuncture site for chelonians. *J. Herp. Med. Surg.* **12**, 32–37.

Hunter Jr., K.W., duPré, S.A., Sharp, T., Sandmeier, F.C. and Tracy, C.R. (2009) Western blot can distinguish natural and acquired antibodies to *Mycoplasma agassizii* in the desert tortoise (*Gopherus agassizii*). *J. Microbiol. Meth.* **75**, 464-471.

Jacobson, E.R. (2007) Viruses and viral diseases of reptiles. In: Jacobson, E.R. (Ed.) Infectious Diseases and Pathology of Reptiles. CRC Taylor and Francis Group, New York, pp. 395-460.

Johnson, A.J., Pessier, A.P., Wellehan, J.F.X., Brown, R. and Jacobson, E.R. (2005) Identification of a novel herpesvirus from a California desert tortoise (*Gopherus agassizii*). *Vet. Microbiol.* **111**, 107-116.

Leslie, G. A. and Clem, L. W. (1972) Phylogeny of immunoglobulin structure and function VI. 17S, 7.5S, and 5.7s anti-DNP of the turtle, *Pseudemys scripta. J. Immunol.* **108**:1656-1664.

Mohammadpour, H.A., Tracy, C.R., Redelman, D., duPre,' S.A. and Hunter, K.W. (2010) Flow cytometric method for quantifying viable *Mycoplasma agassizii*, an agent of upper respiratory tract disease in the desert tortoise (*Gopherus agassizii*). *Lett. Appl. Microbiol.* **50**:347-51.

Pettan-Brewer, K.C.B., Drew, M.L., Ramsay, E., Mohr, F.C. and Lowenstine, L.J. (1996) Herpesvirus particles associated with oral and respiratory lesions in a California desert tortoise (*Gopherus agasszii*). J*. Wildlife Dis.* **32**, 521-526.

Schumacher, I.M., Brown, M.B., Jacobson, E.R., Collins, B.R. and Klein, P.A. (1993) Detection of antibodies to a pathogenic mycoplasma in desert tortoises (*Gopherus agassizii*) with upper respiratory tract disease. *J. Clin. Microbiol.* **31**, 1454-1460.

Snipes, K.P. and Fowler, M.E. (1980) A Pasteurella spp. associated with respiratory disease in captive desert tortoises. *J. Amer. Vet. Med. Assoc.* **177**, 804-807.

Turchin, A. and Hsu, E. (1996). The generation of antibody diversity in the turtle. *J. Immunol*. **156**, 3797-3805.

U.S. Fish and Wildlife Service. (1990) Endangered and threatened wildlife and plants; determination of threatened status for the Mojave population of the desert tortoise. *Federal Register* **55**(**63**), 12178–12191.

U.S. Fish and Wildlife Service. 2011. Revised recovery plan for the Mojave population of the desert tortoise (*Gopherus agassizii*). U.S. Fish and Wildlife Service, Pacific Southwest Region, Sacramento, California. 222pp.

Conclusions

Technical challenges that have impeded critical microbiological studies of *M. agassizii* include its small size which limits the use of light microscopy for cell counting, and its slow growth characteristic in broth and agar cultures which impedes colony counting. The first goal of this project was to develop a rapid and sensitive flow cytometric technique using a vital fluorescent dye for enumerating viable *M. agassizii* cells. Here we show that non-fluorescent 5-CFDA-AM molecules, once internalized by viable *M. agassizii*, are converted into fluorescent 5-CF molecules through esterase hydrolysis and are retained within the cells. This demonstrates that a single fluorescent label can be used to rapidly enumerate viable *M. agassizii* by flow cytometry. The ability to detect and enumerate viable *M. agassizii* in broth cultures by 5-CFDA-AM staining allows researchers to perform a variety of experiments with these organisms in the laboratory which were previously not possible using conventional culture methods.

To further elucidate the natural history of M. agassizii infections, reagents that can detect the major immunoglobulin isotypes of the desert tortoise were needed. We used cross-absorption to generate IgM and IgY heavy chain specific rabbit polyclonal antibody reagents to facilitate the study of humoral immune responses in the desert tortoise. Using a combination of SDS-PAGE and Western blotting, we demonstrated the heavy chain specificity of the reagents, and also showed that the apparent molecular weights of the IgM and IgY heavy chains were 72 kD and 68 kD respectively.

We showed that isotype-specific rabbit anti-tortoise IgM and IgY reagents can be used to follow the antibody response following vaccination, and we further showed the usefulness of these reagents to study the natural course of an infection in the desert tortoise. The changing pattern of antibody reactivity with antigens of *M. agassizii* revealed by Western blot, and the isotype-specificity of this response, provide another tool to assess the seroepidemiology of infectious diseases like mycoplasmosis in the desert tortoise.

Supplemental Data

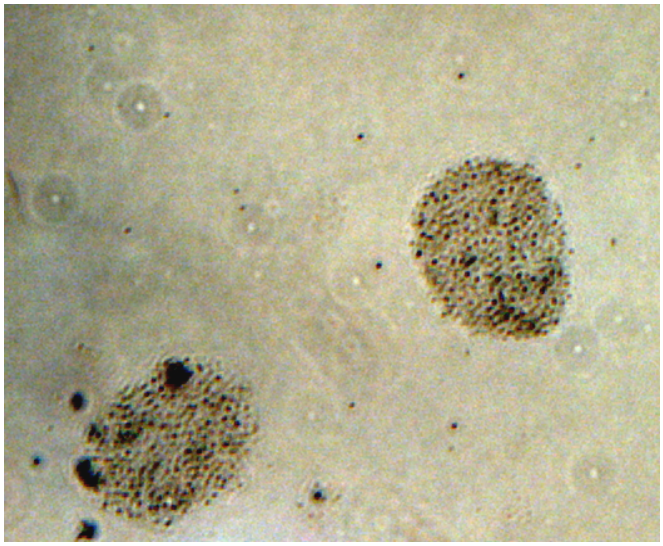

Figure S1. Mycoplasma are less than 300 nM in size preventing visualization using standard microscopy. They are extremely slow growing and colonies take several weeks to form. These colonies of *M.agassizii* grown on SP4 agar are visible after 2.5 months in culture (400X maginification).

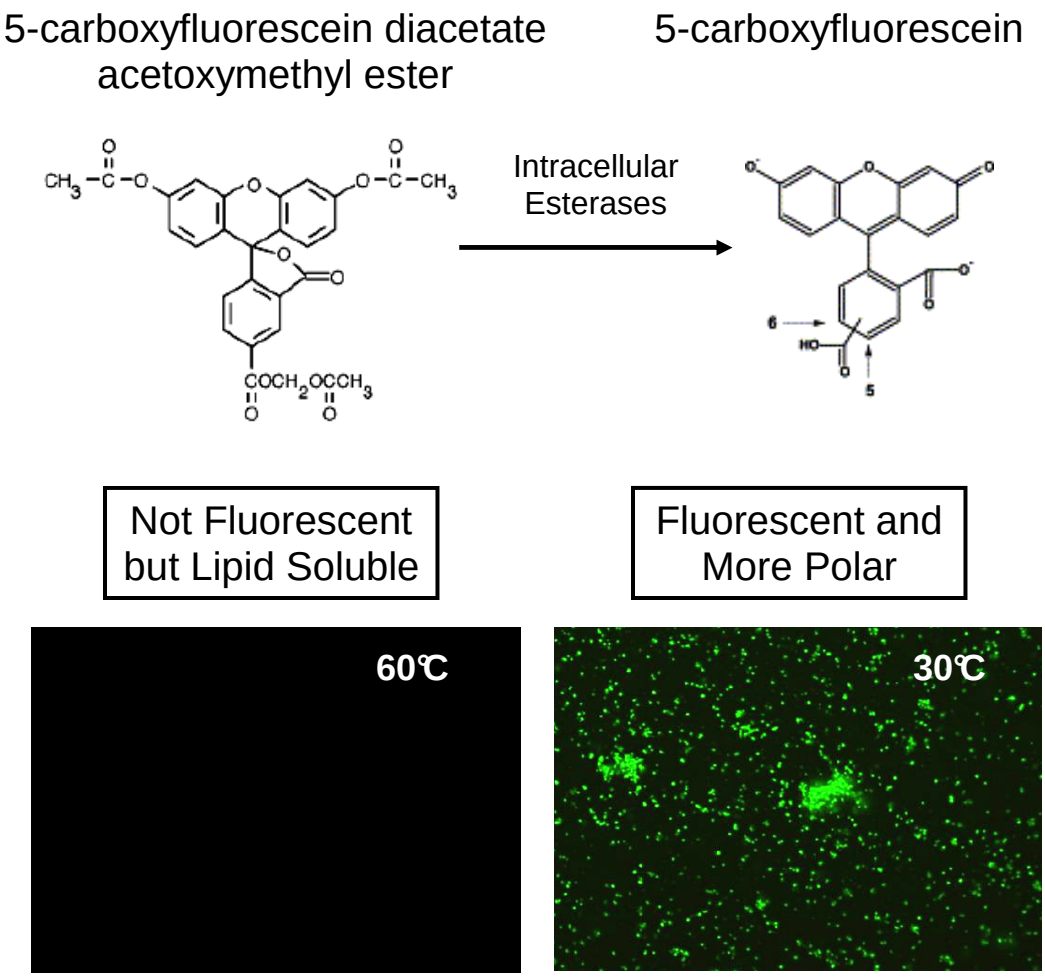

Figure S2. a) Fluorescent activation of 5-CFDA-AM by esterase. 5-carboxyfluorescein diacetate, acetoxymethyl ester (5-CFDA-AM) is a non-fluorescent molecule that is converted to 5-carboxyfluorescein diacetate (5-CF), a fluorescent metabolite, by intracellular esterases found in viable cells. Viable *M. agassizii* were split into two groups. One group was heat inactivated at 60ºC for one hour (image b). The other was incubated at normal culture conditions; 30ºC (image c). Both groups were washed several times with PBS and incubated in the dark, end-over-end, for 1 hr at room temperature in 1:1000 5-CFDA-AM: PBS solution. Cells were centrifuged and washed several times with PBS. Cell pellets were diluted in PBS and aliquots were scanned using confocal microscopy. Heat inactivated *M. agassizii* were unable to convert 5-CFDA-AM into the fluorescent 5-CF (see image b). Untreated *M. agassizii* converted 5-CFDA-AM into the fluorescent 5-CF demonstrating the usefulness of 5-CFDA-AM for visualization and enumeration of viable mycoplasma (see image c).

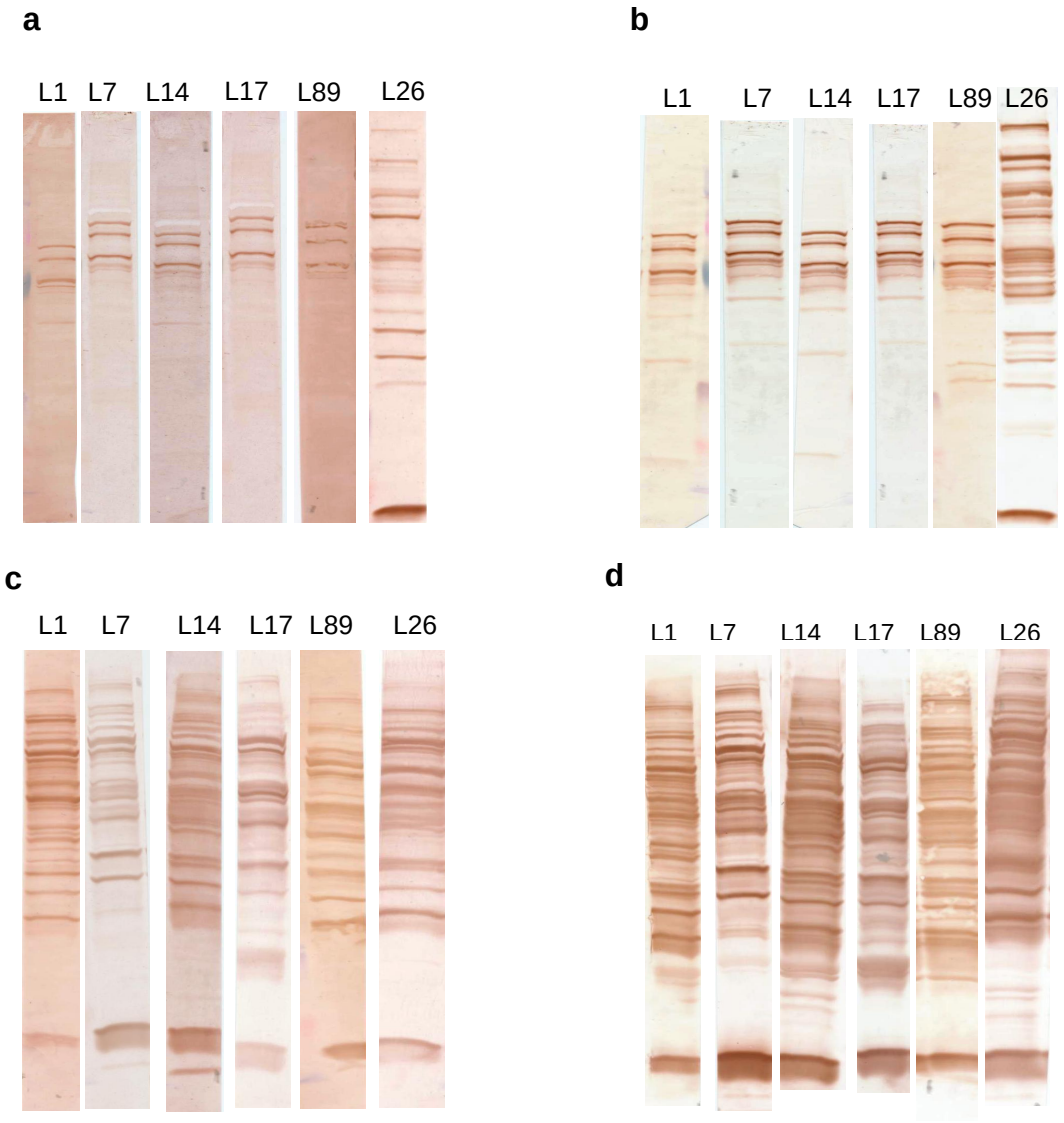

Figure S3. Six desert tortoises were infected with *M. agassizii* as described in Chapter 3. Five of the six tortoises did not initially seroconvert and so only one tortoise (L26) was followed over the course of the study (blot a shows IgM and blot b shows IgY 64 days post infection). Tortoise L26 developed clinical signs and symptoms of URTD three years post infection. This lead us to collect plasma from the remaining five infected tortoises, although they were not demonstrating signs of URTD. Western blot analysis showed that all six tortoises had developed a delayed immune response to the infection and tested positive for IgM and IgY antibodies (blot c and d respectively).