STRESSOR INDUCED IMMUNOMODULATION IN CROCODILIANS

by

JOHN WILSON FINGER JR.

(Under the Direction of Travis C. Glenn)

ABSTRACT

Crocodilians are an ancient lineage composed of long-lived, top trophic carnivores and that are of evolutionary, economic, and ecological importance. Recent advances and anecdotal observations, along with evolutionary insights, have increased interest into the immune system of crocodilians. Immunocompetence, the ability to mount an effective immune response following pathogenic exposure, is of paramount importance in the life history of an organism. However, this ability may be regulated by a number of stressors including infection, toxicant exposure, climate, reproduction, and various other biotic and abiotic factors. Physiological responses to stressors may be varied, including activation of the 'fight or flight' response, alterations in metabolic rate, perturbations in physiological processes, or activation of the hypothalamic-pituitary-adrenal (HPA) axis, however, all responses function to preserve and reinstate homeostasis following stressor stimulation. Whilst the definition of a stressor is usually consensually defined, the definition of the stress response following stressor stimulation is more ambiguous. Some authorities, however, now suggest restriction in defining stress to only those responses that are mediated by the HPA axis culminating in production of

glucocorticoids (GCs). Crocodilians represent an interesting lineage in which to investigate the effects of stressors on the immune system because of their slow evolutionary rate, basal phylogenetic position among archosaurs, role as economic commodities, and high trophic placement. In particular, crocodilians may serve as indicators of habitat quality, thus, examination of how exogenous toxicants affect their immune systems may provide insight into other species and the overall health of the environment. Furthermore, many crocodilians, such as the American alligator, are keystone species, so investigations of stressors and their impact on immune function are necessary for conservation. Some crocodilians, like the saltwater crocodile and alligator, represent important economic commodities providing monetary incentives for conservation through sustainable use. However, such increased interaction may increase susceptibility to zoonoses. In this dissertation, I investigate the immune system of crocodilians and how stressors may influence the immune system through the action of toxicants, microbial disease, and agricultural production settings.

INDEX WORDS: Crocodilian, Stressor, Immunology, Toxicology, Agricultural Production

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DEDICATION

This is dedicated to all my ancestors and family members that worked so hard out in the fields, in the mountains, in the mills, in the pharmacy, in the classroom, or in the staffing office so I could follow my dreams and study crocodiles and alligators.

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CHAPTER 1

INTRODUCTION

Overview of Stressors

The ability to mount an effective immune response following antigenic exposure is important in the life history of an organism (Demas et al. 2011; Finger et al. 2013). This ability, termed immune responsiveness (Vinkler et al. 2011), may be regulated by a number of stressors including pathogenic infection, toxicant exposure, climactic factors, reproductive status, and various other biotic and abiotic factors (Hopkins et al. 1997; Moore and Jessop 2003; Shini et al. 2008; Johnstone et al. 2012). Physiological responses (i.e. stress) to stressors may be varied, including rapid activation of the sympathetic-adrenal-medullary (SAM) directed 'fight or flight' response (Selye 1936), alterations in metabolic rate (DuRant et al. 2007), perturbations in certain physiological processes, changes in growth (Ward et al. 2006), or activation of the hypothalamic-pituitary-adrenal (HPA) axis (Johnstone et al. 2012), however, all responses function to preserve and reinstate homeostasis following stressor stimulation (Moore and Jessop 2003; Shini et al. 2008).

Whilst the definition of a stressor is usually consensually defined (at least relative to the response), the definition of the stress response (i.e. what stress actually is) following stressor stimulation is more ambiguous (Selye 1976; Johnstone et al. 2012). Stress was first defined as a nonspecific response induced following exposure to a stressor, that is, any 'nocuous agent' that was capable of inducing a stress response (Selye

1936; 1976), however, some now suggest restriction in defining stress to only those responses that are mediated by the HPA axis and stressors as stimulators inducing HPA activation (Johnstone et al. 2012). During such an HPA mediated response, hypothalamic parvocellular neurons release corticotrophin-releasing hormone (CRH) following stimulation into the hypophyseal portal system. CRH subsequently induces release of adrenocorticotropic hormone (ACTH) from anterior pituitary corticotrophic cells, promoting production and secretion of the glucocorticoids (GCs) from the adrenal cortex (Norris 2007). GC secretion subsequently increases lipolysis and proteolysis in target cells, providing gluconeogenic precursors for stressor alleviation. Along with increased blood flow to necessary organs (i.e. brain), bronchodilation (i.e. increased oxygen intake), and other physiological effects (i.e. reduced blood flow or protein catabolism in non-essential organs), these precursors can be then converted to glucose in the liver and subsequently routed throughout the body to aid in mitigation of stressors (Norris 2007). Whilst such responses are initially protective in nature such as promoting escape behaviors, reproduction, or enhancing immune function (see Dhabhar 2002; Romero 2002), prolonged stimulation may induce physiological suppression. However, not all types of stressors elict a response via the HPA axis. In fact, some environmental stressors, such as environmental toxicants, may induce deviations in other processes, such as alterations in endogenous hormones, gamete production, embryonic development, or immune function (Guillette et al. 1994; Guillette et al. 1997; Burnham et al. 2003). However, some toxicants may affect the HPA axis and consequently, the production of GCs (Hopkins et al. 1997).

As discussed above, the definitions of ‘stressor’ and ‘stress response’ are quite contentious. In this dissertation to encompass a wide array of stressors placed upon crocodilians, we will define a stressor in a more general sense similar to that of Selye (1936), in that a stressor is any perceived nocuous agent (environmental, physiological, or psychological) capable of inducing changes in homeostasis and eliciting a stress response (Peterson et al. 1991).

In this dissertation, we will focus on reputable nocuous agents (i.e. stressors) and their influence on the immune system. Firstly, we will review the innate immune functions of crocodilians to provide a basis of knowledge on the subject. Secondly, we will explore how toxicants, such as endocrine disrupting compounds, may affect the immune system through insights from other species. Thirdly, we will discuss a pervasive *in vivo* technique that we developed to investigate immune function in crocodilians and that was employed in subsequent chapters. Fourthly, we will investigate the susceptibility of crocodilians to pathogenic microbes, such as influenza virus. Fifthly, we will examine the effect of agricultural production settings on crocodilians and the consequent impacts on the immune system. And finally, we will examine how chronic exposure to anthropogenic toxicants may affect the immune system in crocodilians.

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CHAPTER 2

LITERATURE REVIEW: A REVIEW OF INNATE IMMUNE FUNCTIONS IN CROCODILIANS [1]

[1] Finger, J.W. Jr., and S.R. Isberg. 2012. *CAB Reviews*. 7, 67:1-11. doi: 10.1079/PAVSNNR20127067. Reprinted here with permission of the publisher

Abstract

The immune function of crocodilians is understudied but is of interest for medical, ecological and evolutionary purposes. Crocodiles share a common ancestor with birds, comprising the archosaurian lineage, so they are an important link in our understanding of immune system evolution. As top trophic carnivores, they inhabit temperate and tropical climates in their semi-aquatic environment. However, they are also ectothermic, whereby environmental temperatures affect their physiological processes, including immune function, adding to the complexity of research in this area. Anecdotal observations and recent research have augmented enthusiasm in the realm of crocodilian immunology. Despite comprising both adaptive and innate systems, most research has investigated the innate system, which comprises peptides, proteins and leucocytes functioning in defence. Herein, we provide an overview into the innate immune system of the crocodile and areas for further research.

Introduction

The study of crocodilian immunology is in its infancy. However, recent research and a multitude of anecdotal evidence has suggested potent immune capabilities, and necessitated a greater understanding of the immunology in this ancient group of archosaurian reptiles (Merchant et al. 2003; 2004; 2005). Crocodilians comprised 23 different species divided into three different families: Crocodylidae, Alligatoridae and Gavialidae, with true crocodiles comprising Crocodylidae, alligators and caimans encompassing Alligatoridae, and gharials comprising Gavialidae (Brochu 2003). Crocodilians are opportunistic, top trophic carnivores inhabiting a semi-aquatic environment. Similar among all non-avian reptiles, crocodilians are ectothermic, and thus

lack the necessary high metabolic rate and insulation for endothermic (Coulson and Herbert 1981; Seebacher et al. 2005). As such, all physiological processes are directly entwined with temperature, including immune function, feeding and assimilation, reproduction, and growth and development (Lance 2003; Lang and Andrews 1994). Thus, for optimal physiology, crocodilians must regulate their body temperature through behavioural means (i.e. basking and submergence; Smith 1975). As in all jawed vertebrates, the crocodilian immune system is dichotomous, composed of both innate and adaptive (acquired) portions (Dzik 2010). Innate functions are present from birth (germline encoded), acute in their onset, require no previous stimulation and are non-specific, whereas adaptive functions are specific in nature, non-germline encoded (arise via somatic recombination) and require previous stimulation with subsequent infections/exposures increasing efficacy.

The purpose of this review is to provide an insight into the overall immunobiology of crocodilians, with the hope of revealing the gaps for continued research. Although the presence of the adaptive system has been documented in crocodilians able to produce both T and B lymphocytes and exhibiting immunological memory, most of research has investigated the innate immune system (Cuchens and Clem 1979; Lerch et al. 1967). Thus, discussion will mainly be restricted to innate aspects of the crocodilian immune system, although we realize the necessity of continued research into both components to fully elucidate the mechanisms of these two interdependent systems.

Lymphoid Tissues in Crocodilians: Structure and Hormonal Regulation

Crocodilian, as with other reptilian, lymphoid tissues consist of the spleen, thymus, bone marrow and gut- associated lymphoid tissue (GALT), but are devoid of germinal centres and lymph nodes (Table 2.1) (Solas and Zapata 1980; Zimmerman et al. 2010). Analogous to hibernating mammals, reptilian lymphoid tissues exhibit involution during certain times of the year with functionality varying between changing seasons and temperatures (El Ridi et al. 1988; Zapata et al. 1992). Given this variability, ascertaining lymphoid organ functionality and overall immunocompetence adds additional complexity. For example, temperature has been shown to affect haematology values of captive saltwater crocodiles, *Crocodylus porosus*, although interestingly there was no effect on plasma immunoglobulin levels in the same animals (Turton et al. 1997). Similarly, innate and adaptive immune components exhibit seasonal variation in slider, *Trachemys scripta*, and painted turtles, *Chrysemys picta* (Schwanz et al. 2011; Zimmerman et al. 2010). Thus, depending on season and temperature (and a host of other abiotic factors), immune functionality may display differing parameters.

Much of this seasonality in immune parameters may be the result of endogenous hormones, as some studies have shown quantitative changes in leucocyte numbers and lymphoid involution to be associated with changes in circulating levels of glucocorticoids and sex steroids (Dai et al. 2009; Daviz et al. 2008; Morici et al. 1997; Ruiz et al. 2010; Turton et al. 1997; Zapata et al. 1992). In addition, exogenous exposure to hormonal mimics, such as endocrine disrupting chemicals (EDCs), may also affect immunity. The susceptibility of crocodilians to EDCs has previously been elucidated as they are top trophic carnivores, so EDCs may bioaccumulate and biomagnify in tissues, vastly

affecting normal physiology (Guillette et al. 1994; 1996; Milnes and Guillette 2008; Rey et al. 2009; Stoker et al. 2003). Crocodilian exposure to EDCs has also been associated with a number of immune abnormalities including histological alterations in immune organs (Rooney et al. 2003).

By convention, lymphoid tissues are classified into primary and peripheral lymphoid tissues. Primary lymphoid tissues are sites of lymphocyte production, whereas peripheral organs facilitate innate and adaptive interaction and promote clearance of pathogens (Pabst 2007). As in most vertebrates excluding birds, the thymus and bone marrow function as primary tissues in crocodilians from which mature cells disperse and interact with pathogens in peripheral tissues (Scollay et al. 1980). During reptilian embryonic development, haematopoiesis mainly occurs in the yolk sac, whereas upon hatching, the bone marrow serves as the primary site of haematopoiesis (El Deeb et al. 1985). However, other organs, such as the thymus, spleen and liver, may also play an important role in haematopoiesis (Jacobson 2007). Immature T-cell progenitors migrate out of the bone marrow and finish maturation in the thymus, while B-cell maturation continues in the bone marrow. It is from these primary tissues that naive (never encountered antigen) lymphocytes disperse and engage pathogens in peripheral lymphoid tissues. Contrastingly in birds, the closest living relative of crocodilians, maturation of B-cells does not occur in the bone marrow but in an avian-specific organ located near the cloaca, the bursa of Fabricius (Warner and Szenberg 1964).

Similarly, among vertebrates, the crocodilian thymus is composed of an outer cortex and inner medulla, with connective tissue septa partitioning the thymus into discrete lobules (Rooney et al. 2003). Histologically, the outer cortex shows a higher

concentration of T-lymphocytes, mediators of cell-mediated adaptive immunity, than the inner medulla (Saad and Zapata 1992). In mammals, T-cells mature as they traverse through the cortex and into the medulla, although this is yet to be investigated in crocodilians (Rooney et al. 2003).

The spleen, a peripheral lymphoid organ, aids in removal of blood-borne pathogens and senescent erythrocytes. Histological staining reveals distinctive splenic compartments, including the red and white pulp and the splenic capsule. The splenic capsule encapsulates the organ itself, while the red pulp comprised reticulated splenic cords and venous vessels (Tanka and Elsey 1997). In mammals, the white pulp comprised splenic follicles, the periarteriolar lymphoid sheath and the marginal zone, which serves as a type of boundary between the red and white pulp (Cesta 2006). In the American alligator, *Alligator mississippiensis*, the white pulp consists of nodular lymphoid tissue surrounding terminal arterioles and sheathed capillaries (Tanaka and Elsey 1997). Although germinal centres arise in mammals, they are not present in the crocodilian spleen (Tanaka and Elsey 1997). In mammals, antigenic stimulation activates B-cells inducing germinal centre formation. These serve as sites for somatic hypermutation and affinity maturation of antibodies, with subsequent differentiation into B-lymphocyte effectors, memory and plasma cells (Crotty and Ahmed 2004; Klein and Dalla-Favera 2008; Schlomick and Weisel 2012). Somatic mutation, affinity maturation and lymphocyte differentiation enhance the adaptive immune response, helping rid the body of the invader. Memory cells provide enhanced responsiveness in subsequent infections, while plasma cells produce copious amounts of antibodies. Thus, the lack of germinal centres, along with lymph nodes, in crocodilians has led some to suggest that the spleen

may play a more important role in immune defence, although further research is obviously needed to elucidate and understand antigen presentation and subsequent B-cell activation in crocodilians (Rooney et al. 2003).

Proteins Functioning in Defence

Innate immunity is the first line of defence against microbial invaders, consisting of barriers (i.e. skin, gut mucosa, etc.), innate leucocytes, as well as antimicrobial peptides (AMPs), enzymes and proteins. Of particular notoriety among antimicrobial enzymes are lysozymes, which in mammals are components of saliva, tear and nasal secretions exhibiting bactericidal activities against both Gram-negative and Gram-positive bacteria (Beutler 2003; Murphy et al. 2007). These enzymes function to hydrolyse the outer components of microbial membranes, namely the glycosidic bond between N-acetylmuramic acids and N-acetyl-glucosamines of bacterial peptidoglycans (Callewaert and Michiels 2010). This enzymatic-mediated hydrolysis allows for the destruction of cellular membrane, subsequent microbial osmotic lysis and eventual clearance of the pathogen. Similar to the documentation of these enzymes in birds, lysozyme-like proteins have also been putatively identified in the egg white and leucocytic extracts of the Siamese crocodile, *Crocodylus siamensis* (Maxwell and Robertson 1998; Pata et al. 2007; Prajanban et al. 2010).

Another antimicrobial enzyme so far identified in crocodilians includes phospholipase A2 (PLA2), which is an antibacterial enzyme catalysing the hydrolysis of phosphoglycerides via the sn-2 acyl bond, and is expressed in both cytosolic and secreted forms (Schaloske and Dennis 2006). The cationic character of PLA2 permits affinity for anionic bacterial membranes, consequently enabling membrane destruction via increased

permeabilization (Buckland and Wilton 2000). Serum extracts have revealed PLA2 in a host of crocodilians including *C. porosus*, *A. mississippiensis*, *C. siamensis*, *Crocodylus niloticus*, *Mesticops cataphractus* and *Osteolameous tetraspis* (Merchant et al. 2009a; Merchant et al. 2011; Nevalainen et al. 2009). For example, to determine the activity of PLA2 in *A. mississippiensis*, Merchant et al. (2009a) treated fluorescently labelled (at sn-2 acyl bond) *Escherichia coli* with alligator serum. Fluorescence signified phosphoglyceride cleavage and consequently denoted PLA2 activity, as this particular enzyme hydrolyses this bond. The use of a PLA2 inhibitor decreased fluorescence, further illustrating the effects of cleavage are mediated by PLA2. Activity, which was both temperature and concentration dependent, was observed within 5 min of incubation, with peak activity observed after 20 min and at 40˚C (Merchant et al. 2009a).

The antimicrobial enzyme dipeptidyl peptidase IV (DPP4), also known as CD26, has also been demonstrated in crocodilians (Merchant et al. 2009b). As a serine ectoprotease, this enzyme has been shown to be important in a number of different physiological processes in mammals, particularly immunity (Kubota et al. 1992). DPP4/CD26 is expressed in both membrane and secreted forms, as well as on the surface of T-cells suggesting that it may play an important role in T-cell activation (Morimoto and Schlossman 1998; Reischer 1994). Other data suggest that DPP4/CD26 activates and regulates the complement system (Shinosaki et al. 2002). In addition, studies with CD26 knockout mice illustrate that DPP4/CD26 is an important mediator of cellular maturation and migration, production of cytokines and various aspects of humoral immunity (Yan et al. 2003). To determine the activity of DPP4 within a crocodilian, Merchant et al. (2009b) incubated both whole blood and plasma from *A. mississippiensis* with the DPP4

fluorogenic substrate Ala-Pro-AFC. Subsequent proteolytic product formation, and thus DPP4 activity, was measured through the amount of fluorescence emitted. DPP4 activity was higher in whole blood when compared with plasma, possibly due to increased production, catalytic efficiency or presence of membrane forms on T-lymphocytes (Merchant et al. 2009b). Both whole blood and plasma displayed temperature dependence, with peak production occurring in the whole blood and plasma at 30 and 40°C, respectively (Merchant et al. 2009b). So, while DPP4 has been identified within the blood and serum of crocodilians, much remains to be determined about the activity and function of this enzyme in terms of crocodilian immunology.

AMPs

AMPs, comprised up to 45 amino acid residues, are an ancient component of innate immunity found across divergent taxa, with most functioning to increase microbial membrane permeability (Andreu and Rivers 1998; Boman 2003; Hancock and Lehrer 1998). AMPs are synthesized as precursor proteins, requiring post-translational modification for activation (Zasloff 2002). Many are cationic and amphipathic, allowing for increased affinity with microbial membranes (Hancock and Lehrer 1998). Consequently, increased membrane attraction results in augmented membrane dysregulation, inducing osmotic lysis. Structurally, AMPs are assembled into distinct groups based on their conformation, including linear peptides lacking cysteine residues, peptides with disulfide bridges enabling β-sheet conformations, and linear peptides with an extraordinary amount of trypto- phan, proline or histidine residues (Boman 2003; Hancock and Lehrer 1998). Although AMPs are well known for their microbial membrane permeabilization abilities, they may also serve as antagonists for pathogen-

specific enzymes or trigger production of antimicrobial substances, further assisting in clearance of pathogens (Andreu and Rivas 1998).

Defensins and cathlecidins are two groups of AMPs produced in leucocytes that aid in pathogenic defence. Defensins are cationic peptides characterized by multiple β-sheet folds and three disulfide linkages. Functionally, defensins act to increase microbial membrane permeability, whereas cathlecidins are a group of cationic, mostly linear AMPs inducing membrane lysis in microbes (Ganz 2003; Zanetti 2004. In addition to membrane permeabilization, cathelicidins may also serve as immunmodulators and function in leucocytic chemotaxis, directing immune responders to sites of infection (da Silva et al. 2009; Zanetti 2004). As of yet, neither defensins nor cathlecidins have been described in crocodilians, though defensin-like proteins and cathlecidins have been observed in the European pond turtle, *Emys orbicularis*, and in the venom of elapid snakes, respectively (Stegemann et al. 2009; Wang et al. 2008). Evidence suggests that defensins and cathlecidins are phylogenetically conserved, with analogues identified in fish, reptiles, birds, mammals and even plants (Wang et al. 2008). Thus, one may posit their existence in crocodilians, although further research is needed for verification. Although cathelicidins and defensins are yet to be found within crocodilians, other AMPs have been identified. Merchant et al. (2006) demonstrated that the leucocytic extracts of *A. mississippiensis* exhibit anti-bacterial, anti-viral and anti-fungal activity. Results from zones of inhibition assays revealed that extracts were effective at inhibiting the growth of six out of the eight *Candida* species tested, all Gram-negative species tested (n=7), with greatest activity observed against *Shigella flexneri* and *Citrobacter freundii*, a number of Gram-positive *Streptococcus* bacteria (2 out of 4), and viruses,

herpes simplex virus-1 and human immunodeficiency virus-1. Antimicrobial activity exhibited heat stability, was not affected by EDTA concentration, and sensitive to protease treatment (Merchant et al. 2006). As proteins are sensitive to EDTA concentration and heat, this suggested that the observed antimicrobial processes were mediated via cationic AMPs.

Pata et al. (2011) recently identified four previously uncharacterized AMPs, termed leucrocins, in leucocytic extracts from *C. siamensis*. Leucrocin I and II demonstrated strong antibacterial activity against *Staphylococcus epidermis*, while Leucrocin III and IV were most effective at inhibiting the growth of *Vibrio cholerae*. In addition, Leucrocin I and II were shown to be effective in the lysis of human erythrocytes and scanning electron microscopy revealed the antimicrobial properties were mediated via permeabilization of microbial membranes (Pata et al. 2011).

Crocosin, another crocodilian-specific antibacterial compound identified in the plasma of *C. siamensis*, has also demonstrated antibacterial activity against both Gram-positive and Gram-negative bacteria (Preecharram et al. 2010). Characteristic of all AMPs, crocosin demonstrated thermostability; however, the compound was impervious to protease digestion, leading the authors to suggest that it may be a possible AMP derivative or composed of other substituents instead of amino acids (Preecharram et al. 2010). Further study is needed to determine the basis of this antimicrobial activity.

The Complement System of Crocodilians

The complement system consists of a group of about 30 soluble plasma proteins that function in host defence. Initially named for their complementary role with antibodies in ridding the body of invaders, these phylogenetically conserved proteins

regulate various aspects of adaptive immunity, aid in microbial opsonization, and promote destruction of invaders through the formation of the membrane attack complex (MAC; Bohana-Kastan et al. 2004; Holland et al. 2002). Opsonization increases phagocytic efficacy, while MAC directly destroys microbes through the formation of pores, triggering subsequent osmotic lysis. Complement proteins are synthesized as inactive precursors (zymogens), with activation occurring through proteolytic cleavage. These cleaved protein products may collectively associate to form convertases, act as opsonins for phagocytic cells or function as anaphylatoxins, the latter of which function in promotion of inflammation (Trouw and Daha 2011). Complement activation proceeds through three distinct pathways including the classical (CP), alternative (AP) and lectin pathway (LP) (Figure 2.1).

Although the mechanism of induction differs among these three pathways, they similarly functionally converge in the formation of complement protein C3 products, C3a and C3b (Trouw and Daha 2011). Important as an inducer of inflammation, C3a acts as an anaphylatoxin, while C3b functions as a component of C5 convertase, the next enzyme in the cascade (Trouw and Daha 2011). Complement protein C5 serves as substrate for C5 convertase, producing C5a and C5b. While C5a promotes inflammation, interaction of C5b with proteins C6, C7 and C8 on the surfaces of microbial membranes induces polymerization and subsequent indention of multiple C9 molecules into microbial membranes (the MAC), triggering osmotic lysis (Bohana-Kastan et al. 2004).

Activation of the CP consists of complement protein C1q binding the Fc receptor of antibody–antigen complex or C-reactive proteins (Murphy et al. 2007). C1q then interacts with other proteins of the C1 complex, C1s and C1r, which possess serine

protease activity. Subsequently, these proteins sequentially cleave C4 into C4a and C4b and C2 into C2a and C2b (Bohana-Kastan et al. 2004). Mannose residues present on microbes function as ligands, recognized by mannose-binding lectin (MBL), for the LP. Ligand-MBL binding activates MBL-associated serine proteases (MASPs), which sequentially cleave C4 and then C2, with the protein products summing to generate C3 convertase, further activating downstream mediators of the complement cascade (Degn et al. 2007). Activation of the AP is achieved via the spontaneous cleavage of an internal thioester linkage in the C3 molecule; production and interaction of C3b with Factor B, a homologue of C2, and subsequent cleavage of Factor B forms C3 covertase C3bBb (Murphy et al. 2007). Amplified production of cleavage product C3b allows for association with C3bBb, forming C5 convertase C3bBbC3b (Harboe and Mollnes 2008; Holland and Lambris 2002). The presence of the AP and complement protein C3 has previously been demonstrated in the serum of the American alligator, *A. mississippiensis* (Merchant et al. 2005). Lysis of exogenous erythrocytes was observed in the presence of CP inactivators (ammonium hydroxide and methylamine), with activity being heat-labile (AMPs are heat-stable), as the prevention of lysis occurred when serum was heated at 56°C for 30 min, thus suggesting that lysis was induced by complement proteins activated via the AP (Merchant et al. 2005). Similar findings were observed in the American crocodile (*Crocodylus acutus*), broad-snouted caiman (*Caiman latirostris*), freshwater crocodile (*Crocodylus johnstoni*) and *C. porosus* (Merchant et al. 2005; 2006; 2010; Siroski et al. 2010). Expectedly, temperature influenced haemolytic activity in each of the species studied. As shown in Figure 2.2, both higher and lower temperatures reduced efficacy of haemolysis, with highest activity observed between 20 and 35°C.

Although these studies demonstrate that crocodilians possess the AP for complement activation, to our knowledge, the other avenues of activation have not yet been elucidated, providing a gap in our understanding of this system in crocodilians.

As in mammals, crocodilian complement proteins may act as opsonins, enhancing phagocytic efficacy. Aree et al. (2011) demonstrated the opsonizing effects of crocodilian serum via incubation of murine macrophages and two types of bacteria, *Staphylococcus aureus* and *E. coli*, in the presence of *C. siamensis* serum. Incubation with serum induced 2- and 4-fold increases in phagocytic uptake of *S. aureus* and *E. coli*, respectively, while pre-treatment heating of serum reduced this phagocytic ability (Aree et al. 2011).

Innate Leucocytes

Leucocytes or white blood cells (WBCs) are important mediators of the immune system, carrying out the tasks of sensing and ridding the body of pathogenic microbes. WBCs arise from pluripotent haematopoetic stem cells located within the bone marrow, which further differentiate into common lymphoid and common myeloid progenitors. Myeloid cells include erythrocytes, thrombocytes, granulocytes and monocytes, while natural killer cells and the lymphocytes, B- and T-cells (adaptive cells) are members of the lymphoid lineage. Lymphocytes possess specific receptors owing to the events of somatic recombination, while innate leucocytes express germline encoded, non-specific, phylogentically conserved receptors termed pattern recognition receptors (PRRs), which recognize conserved molecules or molecular patterns on pathogenic microbes, subsequently inducing production of inflammatory mediators, antiviral molecules (interferons), chemotactic factors and phagocytosis, depending on cell type (Hoffman et al. 2001; Kawai and Akira 2006; Kimbrell et al. 2001).

The innate leucocytes of crocodilians include heterophils, eosinophils, basophils and monocytes (Canfield 1985; Glassman et al. 1981; Mateo et al. 1984; Zayas et al. 2011). Although the phagocytic azurophil has been identified in squamates (lizards and snakes), its presence remains uncertain within the crocodilian vasculature (Montali 1988). However, some haematological studies have observed staining representative of azurophils in the Nile crocodile, *C. niloticus*, and Morelet's crocodile, *Crocodylus moreletti* (Lovely et al. 2007; Padilla et al. 2011). Given the presence of numerous cytoplasmic granules (which influence staining characteristics), heterophils, eosinophils and basophils are collectively termed granulocytes. In mammals, granulocytes are also referred to as polymorphonuclear leucocytes because of the multi-lobed appearance of the nuclei, whereas the nuclei of reptilian granulocytes are more heterogeneous in appearance and may appear round (non-segmented), multi-lobed or bi-lobed (Montali 1988).

Heterophils are the functional equivalent of mammalian neutrophils, and as such, function as phagocytes. However, unlike mammalian neutrophils, crocodilian heterophils stain eosinophilic, possessing fusiform-shaped eosinophilic cytoplasmic granules (Jacobson 2007; Maxwell and Robertson 1998). Heterophils comprise more than 50% of the circulating leucocytes in *A. mississippiensis*, although quantitative differences may arise because of other factors, such as infection, seasonality or age (Glassman et al. 1981 Mateo et al. 1984). The nuclei of these cells are oval in appearance, lacking the multi-lobed appearance characteristic of mammalian neutrophils (Glassman et al. 1981 Mateo et al. 1984). Similar to mammalian neutrophils, heterophils are the first cells to arrive at the site of infection, followed by mononuclear phagocytes (Jacobson 2007). For example,

Mateo et al. (1984) found that alligator heterophils infiltrated damaged tissue within 4 h following administration of turpentine.

Eosinophils and basophils make up about 10 and 12% of circulating leucocytes in crocodilians, respectively (Mateo et al. 1984). Although both cell types are imperative in regulating the inflammatory milieu, eosinophils may also assist in parasitic infections, with eosinophilia (an increase in eosinophil numbers) commonly observed during helminth-associated infections in mammals (Behm and Ovington 2000). Similarly, in *A. mississippiensis* eosinophilia has also been observed in response to leech infections (Glassman et al. 1979). Basophils are innate cells commonly implicated in allergic reactions, releasing pro-inflammatory mediators, such as histamine, from cytoplasmic granules following antibody-Fc receptor binding (Gibbs 2005; Sullivan and Locksley 2009). Important innate phagocytic cells include the mononuclear phagocytes, comprising monocytes, macrophages and dendritic cells (DCs; Beutler 2004). Aptly termed antigen-presenting cells, these cell types function in linking innate and adaptive immunity through antigen presentation and subsequent activation of adaptive responses. In *A. mississippiensis*, monocytes comprise about 1% of peripheral leucocytes (1.5+SD 0.3), whereas in *C. porosus* yearlings, the range (4.5–21.6) was much greater (Mateo et al. 1984; Millan et al. 1997). In mammals, these cells give rise to tissue-associated macrophages and some types of DCs (Geissmann et al. 2010; Gordon and Taylor 2005). Macrophages are important in a number of physiological processes including ingestion of senescent erythrocytes, tissue repair, disposing of apoptotic cells, and notably, ‘eating’ invading microbes (Stuart and Ezekowitz 2005). In mammals, many subsets of DCs have been identified including plasmacystoid DCs, producers of copious amounts of interferon

aiding in defence against viruses, and Langerhans cells, epidermal cells important in the transport of ingested microbes to lymph nodes for antigen presentation (Naik 2008; Romani et al. 2003). However, in reptiles, notably crocodilians, characterization and understanding of DCs remain scanty, although the presence of such cells has been noted in snakes (Kroese et al. 1985). Understanding of these cell types could provide further insights into how the adaptive immune response is regulated.

Phagocytosis of pathogenic microbes requires participation of PRR–ligand interaction, stimulating ingestion and formation of a membrane-enclosed vesicle containing ingested microbe, the phagosome (Lee and Kim 2007). Following ingestion, the phagocytic vesicle is acidified, subsequently fuses with lysosomes, establishing the phagolysosome, composed of proteases and a host of antimicrobial substances (Murphy et al. 2007). Further, ingestion stimulates the production of reactive oxygen species (ROS), the so-called oxidative burst, through the activation of NADPH oxidase, an enzyme complex assembled on the phagosome membrane (Babior 1984; Slauch 2011). NADPH oxidase initially acts to reduce molecular oxygen (O_2) producing superoxide anion (O_2^-), the basis for all phagosome-derived ROS (El Benna et al. 2005). Subsequent dismutation of O_2^- produces hydrogen peroxide (H_2O_2), which may further react with iron, via the Fenton reaction, to produce hydroxyl radical ($OH^\cdot$), a potent oxidizing agent capable of inducing alterations in biomolecules (Babior et al. 1984; El Benna et al. 2005; Slauch 2011). Although the exact mechanisms underlying the bactericidal activities of the oxidative burst are unknown, production of ROS is thought to be of paramount importance (Slauch 2011).

Leucocytic production of ROS has been documented within crocodilian phagocytes (Merchant et al. 2009c). To demonstrate ROS production within crocodilian leucocytes, Merchant et al. (2009c) incubated whole blood from *A. mississippiensis* with water-soluble tetrazolium salts to determine spectrophotometrically the amount of O_2^- produced through the formation of reduced formazan compounds. Leucocytic production of O_2^- demonstrated temperature, concentration and time dependence, respectively. Peak production of O_2^- was observed at 30 and 35°C. Production increased up until 48 h post initial incubation, with no observed statistical differences in product observed after 48 h. Staining of *A. mississippiensis* leucocytes indicated the principal producers of O_2^- to be heterophils and monocytes (Merchant et al. 2009c). Further investigation may provide insight into how production is regulated and to determine the effect of ROS generation on overall crocodilian immunocompetence.

Fever in Crocodilians

Fever is characterized as a hypothalamic-mediated elevation of body temperature above normal levels, with induction triggered through the action of pyrogenic molecules (Atkns 1982; Dascombe 1985; Molts 1993). Pyrogenic molecules activate cyclooxygenases, consequently increasing prostaglandin E2 synthesis and raising the hypothalamic thermoregulatory set point (Dinarello 2004; Moltz 1993). Fever is phylogenetically conserved, being observed across many distantly related species (Hasday et al. 2000; Kruger 1978). In particular, studies with squamates have demonstrated that bacterial-exposed reptiles are able to mount a febrile response when provided with thermal gradients (Bernheim et al. 1976; Burns et al. 1996).

Evidence suggests that crocodilians may also demonstrate the ability to induce a febrile response, though as in other reptiles, the fevers of crocodilians are behavioural in nature, relying on ambient temperatures for induction of said response (Merchant et al. 2007). This is in contrast to mammalian fevers, which are derived of increased metabolic heat production (Hasday et al. 2000). To determine the febrile response of crocodilians, the body temperatures of *A. mississippiensis* injected with lipopolysaccharide (LPS; membrane component of Gram-negative bacteria) were monitored to determine internal temperature fluctuations. LPS-injected alligators kept at a constant temperature exhibited no change in internal body temperature, while those exposed to thermal gradients demonstrated increased internal body temperatures (4.2 and 3.5°C higher at 1 and 2 days post injection, respectively; Merchant et al. 2007). Thus, immune-challenged alligators, when presented with thermal gradients, were able to select sites for optimal heat gain and induce up-regulation of body temperature, that is, producing a febrile response. Interestingly, intra-peritoneal injections with the Gram-positive bacterium, *S. aureus*, did not induce fever, whereas injections with Gram-negative bacteria did in fact provoke a febrile response (Merchant et al. 2007).

Conclusions

The immune system of crocodilians is currently understudied. Anecdotal observations of conspecific combat resulting in limb amputation or mighty wounds, with combatants rarely seeming to succumb to infection or disease, have stimulated postulation of potent immune capabilities and encouraged exploration of crocodilian immune functions. Although such evidence suggests a heightened immune response, this is unsubstantiated, as our understanding of such observations is not known, requiring

further study for elucidation. The most recent exploration of crocodilian immunity has been restricted to innate immune aspects, an ancient arm of immunity conserved in all metazoans, functioning as a first line of defence against invading microbes and subsequently activating the adaptive immune system via antigen presentation and release of chemical mediators (Merchant et al. 2003; 2004; 2005; Dzik 2010).

In conclusion, we have attempted to provide insight into the innate mechanisms of crocodilian immunity. As we have illustrated, understanding of the innate immune system in crocodilians has increased over the past 30 years, with important cell types, proteins and peptides identified. However, there are still many avenues awaiting exploration. Some have suggested that crocodilians may serve as important indicators of environmental health, as they are top trophic carnivores, with optimal physiology and development perturbed following exposure to exogenous toxicants (Rey et al. 2009). Others have suggested that the potent blood components of crocodilians may prove useful in the realm of human medicine in combating sickness and disease (Pata et al. 2011). In addition, further exploration may enable further understanding of the evolution of immune mechanisms. Thus, increased insight into crocodilian immunity is necessary not only to further knowledge of crocodilian biology, but it may also improve our understanding in toxicology, medicine, ecology and evolution.

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Tables

Table 2.1 Immune tissues and cell types of amniotic vertebrates.

Immune Tissues and Cell types of Amniotic Vertebrates				
Tissue/Cell	**Non-archosaurian reptiles** [13-14,17,31]	**Crocodilians** [11,12,27,31,34,84]	**Birds** [31,32,43]	**Mammals** [31,41]
GALT	+	+	+	+
Thymus	+	+	+	+
Spleen	+	+	+	+
Lymph Nodes	-	-	+	+
Bursa of Fabricius	-	-	+	-
Bone Marrow	+	+	+	+
Heterophils	+	+	+	-
Azurophils	+	?	-	-
Eosinophils	+	+	+	+
Basophils	+	+	+	+
Neutrophils	-	-	-	+
Monocytes	+	+	+	+
Lymphocytes	+	+	+	+

Figures

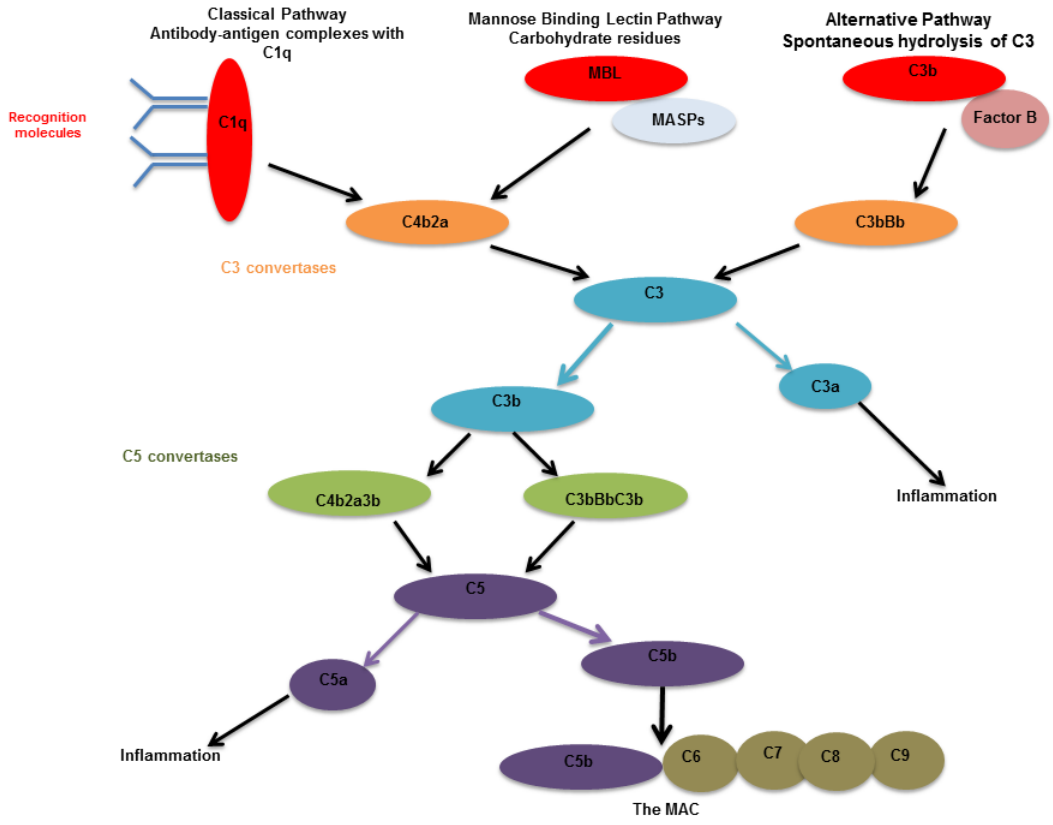

Figure 2.1: Pathways of complement activation.

The complement system may be activated via three distinct pathways including the classical, lectin, and alternative pathway. Classical pathway activation proceeds through C1q binding of antigen-antibody complexes, binding of mannose binding lectin (MBL) to terminal mannose residues present on microbes activates the lectin pathway, whereas the alternative pathway is activated via spontaneous hydrolysis of C3. Only the alternative pathway has been demonstrated in crocodilians (Degn et al. 2007; Merchant et al. 2005; 2010; Siroski et al. 2010).

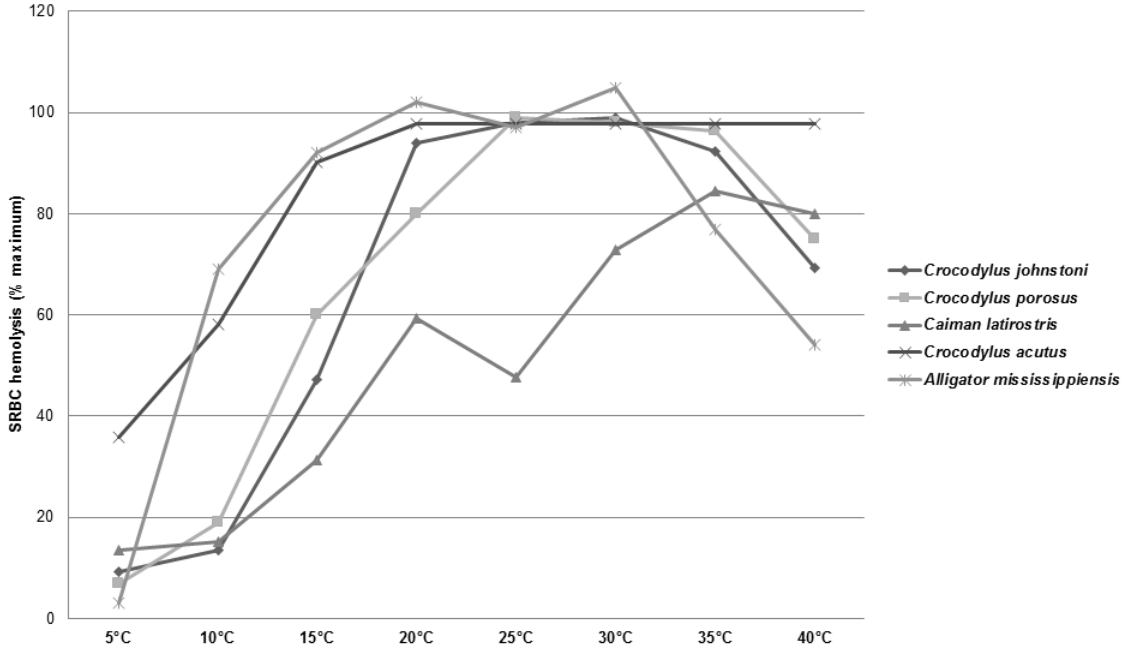

Figure 2.2: The effects of temperature on complement mediated sheep red blood cell hemolysis in five species of crocodilian.

(Merchant et al. 2005; 2006; 2010; Siroski et al. 2010).

CHAPTER 3

ENDOCRINE-DISRUPTING CHEMICAL EXPOSURE AND THE AMERICAN ALLIGATOR: A REVIEW OF THE POTENTIAL ROLE OF ENVIRONMENTAL ESTROGENS ON THE IMMUNE SYSTEM OF A TOP TROPHIC CARNIVORE [2]

[2] Finger, J.W. Jr., and R.M. Gogal Jr. 2013. *Archives of Environmental Contamination and Toxicology*. 65:704-714. doi: 10.1007/s00244-013-9953-x. Reprinted here with permission of publisher

Abstract

Endocrine-disrupting chemicals (EDCs) alter cellular and organ system homeostasis by interfering with the body's normal physiologic processes. Numerous studies have identified environmental estrogens as modulators of EDC-related processes in crocodilians, notably in sex determination. Other broader studies have shown that environmental estrogens dysregulate normal immune function in mammals, birds, turtles, lizards, fish, and invertebrates; however, the effects of such estrogenic exposures on alligator immune function have not been elucidated. Alligators occupy a top trophic status, which may give them untapped utility as indicators of environmental quality. Environmental estrogens are also prevalent in the waters they occupy. Understanding the effects of these EDCs on alligator immunity is critical for managing and assessing changes in their health and is thus the focus of this review.

Introduction

In 1980, a large pesticide spill, composed mainly of estrogenic endocrine-disrupting chemicals [Endocrine-disrupting chemicals (EDCs)], including dicofol and DDT metabolites, occurred in Lake Apopka, Florida. In the years after the spill, EDC contaminants were detected in eggs of American alligators (*Alligator mississippiensis*), and ecological studies showed decreased egg viability and juvenile recruitment in Lake Apopka populations compared with reference lakes (Heinz et al. 1991; Woodward et al. 1993). In a 1994 study, juvenile alligators from Lake Apopka had altered gonadal morphology and serum hormone concentrations suggesting that alligators were affected by the inadvertent release of these EDCs (Guillette et al. 1994). If alligators were shown

to have multiple-organ system sensitivity to EDCs (Figure 3.1), they could function as natural top trophic environmental sentinels (Milnes and Guillette 2008).

Crocodilians are an ancient group of archosaurs, a lineage composed of extant birds and extinct dinosaurs (Meyer and Zardoya 2003). Twenty-three different species have been identified, composed of three distinct familial lineages that diverged in the Cretaceous: the Alligatoridae, the Crocodylidae, and the Gavialidae (Brochu 2003). Some members of the Alligatoridae are found in more temperate climates, such as the American alligator (*Alligator mississippiensis*), a species endemic to the southeastern United States. Resembling all crocodilians, alligators are top-trophic, long lived organisms, and as such they allow for the accumulation of environmental contaminants (Milnes and Guillette 2008). Alligators also modify ecosystems, and these modifications provide habitat for other organisms, especially under times of stress (i.e., drought) (Craighead 1968).

In this article, we first review the role of temperature and hormones in normal crocodilian physiology and then explore various aspects of alligator and reptilian immunity, identifying known immune structures and functions that may be targeted by EDCs. Subsequently, we discuss the role of environmental estrogens in modulating the immune system in various vertebrates and invertebrates and advocate for further study and use of alligators as sentinels for EDC exposure.

Temperature and Hormones in Ectothermic Animals

Alligators, as with all crocodilians, are ectothermic whereby physiological processes are dependent on environmental temperature and behavior used to thermoregulate these processes, such as basking to increase temperature or immersion in water to decrease temperature (Smith 1975). Along with others in the class Reptilia (Elf

2003; Ramsey and Crews 2009), embryonic development, as well as sex, is influenced by (environmental) incubation temperature. Crocodilians exhibit temperature-dependent sex determination (TSD) and display a female–male–female pattern of TSD where females are mainly produced at both low (<32 °C) and high (>33.5 °C) temperatures, and males are predominantly produced in between these temperature ranges (Elf 2003; Ewert and Nelson 2003; Lang and Andrews 1994).

Mechanistically, temperature is thought to modulate endogenous steroid concentrations; in turn these concentrations regulate sex-specific gene expression (Crews 2003; Elf 2003). During development, the alligator gonad is most sensitive to temperature fluctuations [the thermosensitive period (TSP)] between embryonic stages 21 and 24, corresponding to incubation days 30–45 (depending on the temperature), with gonadal differentiation occurring during this TSP (Lang and Andrews 1994; Urushitani et al. 2011). Because the TSP overlaps with steroid sensitivity, shifts in either steroid concentrations or temperature will impact sex determination (Bull et al. 1988; Lance and Bogart 1994; Lang and Andrews 1994; Ramsey and Crews 2009; Wibbels et al. 1991).

TSD can be experimentally altered by way of the administration of select exogenous compounds. A number of studies have shown that treatment of embryonated eggs with exogenous estradiol induces feminization at male temperatures, whereas treatment with aromatase (enzyme converting androgens into estrogens) inhibitors at female temperatures prevents normal ovarian development (Bull et al. 1988; Crain et al. 1997; Gabriel et al. 2001; Lance and Bogart 1994; Milnes et al. 2002). Exogenous estrogen exposure may also impair normal development. As previously mentioned, the Lake Apopka studies illustrate that EDC exposure may modify sex steroid

concentrations, subsequently causing gonadal abnormalities and alterations in gonadal morphology and gene expression (Crain et al. 1997; Guillette et al. 1994; Kohno et al. 2008; Milnes et al. 2008). Furthermore, alligators captured from Lake Apopka were shown to exhibit alterations in splenic and thymic morphology compared with alligators from two reference lakes (Rooney et al. 2003). Because Lake Apopka received years of agricultural runoff and was the site of a large chemical spill (Guillette et al. 1994; Rooney 1998), these alterations may have been due to EDC exposure.

Alligator Immune Function

Environmental temperatures and steroidal hormone concentrations have been shown to influence reptilian immune function (Wright and Cooper 1981; Zapata et al. 1992). For example, reptilian immune responses have been reported to vary among seasons and in response to alterations in hormonal milieus (El Ridi et al. 1988; Zimmerman et al. 2010a). In reptiles, the principal immune tissues include the following: thymus, bone marrow, gut-associated lymphoid tissue, and spleen (Table 3.1) (Zimmerman et al. 2010b). The reptilian thymus, the site of T-cell maturation, exhibits seasonal involution unlike mammals, which increase in size until puberty then atrophy during aging (Zapata et al. 1992; Zimmerman et al. 2010b). The alligator spleen, composed of both red and white pulp, similarly undergoes seasonal involution, a trait also not observed in mammals (Leceta and Zapata 1985; Tanaka and Elsey 1997). Notably, reptiles lack lymph nodes and associated germinal centers, which in mammals are sites of B-cell and helper T-cell interaction, B-cell proliferation, isotype switching, and affinity maturation, all of which associated with increased adaptive immune responsiveness (Guzman- Rojas et al. 2002; Tanaka and Elsey 1997; Vinuesa et al. 2009; Zimmerman et

al. 2010b). The lack of lymph nodes and other peripheral lymphoid tissues characteristic of mammals suggests that the spleen is critical for reptilian immune function (Rooney et al. 2003). Lymphoid tissue involution and degeneration is associated with suppressed immune function and appears to be under hormonal control (El Ridi et al. 1988; Lynch et al. 2009; Zapata et al. 1992). Indeed, in both mammals and reptiles, increased levels of estrogen, by way of administration or endogenous synthesis, are associated with increased thymic atrophy (Hareramadas and Rai 2006; Hince et al. 2008). Thus, EDC exposure, mimicking the action of endogenous hormones, may induce alterations in lymphoid organs by modulating tissue involution. In fact, alterations have been observed in both primary and peripheral lymphoid organs in alligators from estrogen-contaminated sites, although the functional significance of such alterations is not yet well defined (Rooney et al. 2003). Because the immune response is mediated in part by hormones, disruption in the ratios of these hormones may in turn alter alligator immune function. For example, high testosterone levels are necessary for sperm production and breeding. However, at extremely high testosterone levels, decreased immune function and thymic degeneration has been observed in some reptiles (Leceta and Zapata 1985; references within Rooney et al. 2003; Zapata et al. 1992). Thus, EDC exposure, such as to environmental estrogens, could alter testosterone levels (or alter the E/T ratio) resulting in the mobilization of lymphocytes and increasing thymic hematopoiesis. This could shift the immune response toward a proinflammatory response, which could enhance response to self-antigen or even downregulate other immune pathways (Nalbandian and Kovats 2005; Straub 2007).

All species in the animal kingdom possess an innate immune system, whereas only the vertebrate species possess both an innate and adaptive immune system (Dzik 2010). Innate immunity is comprised of various cells and antimicrobial molecules that are nonspecific, recognizing conserved microbial molecular patterns through pattern-recognition receptors. Previous studies indicate that innate immunity is well developed in the alligator (reviewed in Finger and Isberg 2012). The serum contains a wide array of antimicrobial and antiviral components (Merchant et al. 2003, 2004, 2005). As would be predicted with temperature-dependent organisms, the efficacy of the alligator's antimicrobial activity is mediated by temperature, with extreme temperatures diminishing effectiveness (Merchant et al. 2003, 2004). Alligators also possess a potent complement system (Merchant et al. 2003). These plasma proteins recognize and bind pathogenic molecules initiating a cascade of events terminating in lysis or opsonization of pathogen, with the latter undergoing phagocytosis (Bohana-Kashtan et al. 2004; Dzik 2010). Induction of fever has been observed in alligators after injection with either lipopolysaccharide or bacteria (Merchant et al. 2007). Unlike metabolically derived mammalian fevers, febrile responses in alligators are behavioral. Alligators require a thermal gradient to induce fever; thus, they regulate their internal body temperature through selection of sites to maximize heat gain (Merchant et al. 2007). Other antimicrobial enzymes, such as lysozymes, have also been detected in reptiles (Thammasirirak et al. 2006).

Representatives of both myeloid and lymphoid progenitors are present in reptilian tissues (Table 3.2), although an understanding of the immune function of these cells in crocodilians is, for the most part, presumed from other species. Reptilian innate

leukocytes include the following: monocytes, macrophages, heterophils (neutrophil equivalent to mammals), eosinophils, and basophils, with the former three functioning as phagocytes and the latter two important in parasitic infections and inflammation, respectively (Finger and Isberg 2012; Mateo et al. 1984; Sykes and Klaphake 2008; Zimmerman et al. 2010b). In addition, dendritic cells have been identified in the splenic tissue of reptiles (Kroese et al. 1985). In mammals, these are further divided in mammals into myeloid and plasmacytoid dendritic cells, with the latter producing large quantities of interferon cytokines. Interferons are released in response to pathogens and mediate antiviral states in cells, preventing (or attempting to prevent) viral infection and subsequent replication. Molecular extracts from alligator leukocytes exhibit antimycotic and antibiotic activity with evidence suggesting activities are due to antimicrobial peptides (Merchant et al. 2006). In addition to antimicrobial peptides, phagocytic production of reactive oxygen species (i.e., the oxidative burst—functioning in microbial degradation) shows another innate antimicrobial defense identified in alligators (Merchant et al. 2009).

Current knowledge regarding the function of the reptilian adaptive immune system is lacking, although cells of the adaptive immune system, T cells, and B cells have been identified (Cuchens and Clem 1979). Based on studies in mammals, both T cells and B cells originate from a common lymphoid progenitor in the bone marrow and as such are identified as lymphocytes, although they differ regarding their site of maturation and function. B cells, mediators of humoral immunity, respond to extracellular pathogens, whereas T cells, involved in cell-mediated responses, recognize and respond to intracellular pathogens. The lymphoid organs of maturation also differ among the two

cell types, with maturation occurring in the bone marrow for B cells and in the thymus for T cells. T-cell activation requires the involvement of innate mediators, which degrade pathogenic microbes and present peptide antigens by way of major histocompatibility complex (MHC) molecules to T cells. Many T cells, differentiated by markers present on the cell surface (clusters of differentiation [CDs]), have been identified, including cytotoxic, regulatory, gamma delta (cd), and helper T cells. In mammals, CD4+ helper T cells are further divided classically into Th1 and Th2 cells and defined by the cytokines they secrete, with the former mediating more proinflammatory responses and the latter mediating more humoral responses. B cells can recognize intact, soluble (not bound to MHC) antigens. Mammalian B-cell responses have been well characterized; antigen binding to B-cell receptor induces differentiation of cells into antibody-producing plasma cells and memory cells. Unlike mammals, recent evidence suggests that some reptilian B cells may possess phagocytic activity (Zimmerman et al. 2010c). Mammalian plasma cells may produce up to five distinct antibody isotypes, including immunoglobulin IgM, IgE, IgA, IgD, and IgG, with each composed of two identical heavy and two identical light chains with the N-terminal regions of heavy and light chains composed of variable regions (antigen specificity) and the C-terminal (constant) regions regulating other immune functions, including degranulation or phagocytosis on receptor binding. In contrast to mammals, crocodilian B cells may generate up to four isotypes, including IgM, IgY, IgA, and IgD (Magadon-Mompo et al. 2013).

Estrogen Synthesis, Receptors, and Signaling

Estrogens include a group of structurally related cholesterol derivatives. Normal endogenous production is controlled by the hypothalamic–pituitary–gonadal axis with

gonadotropin-releasing hormone inducing the release of the gonadotropins: luteinizing hormone and follicle-stimulating hormone. These gonadotropins induce sex hormone production in the gonads. In the ovaries, the main site of estrogenic biosynthesis, aromatization of androgens produces estrogenic compounds (Meethal and Atwood 2005; Simpson 2003). As soluble steroidal hormones (free or bound), estrogens are able to circulate throughout the body regulating a multitude of tissues including bone, liver, neuronal, reproductive, and immune tissues depending on the location of estrogen receptors (ERs) (Ahmed 2000; Shupnik 2002). Environmental chemicals, such as EDCs, may disrupt normal endocrine physiology and homeostasis by acting as hormonal agonists or antagonists, thus perturbing normal hormone metabolism or affecting hematological transport mechanisms (Milnes and Guillette 2008), such as alterations in synthesis of serum or cytoplasmic sex hormone-binding proteins (Guillette et al. 2000).

Three ERs have been identified in mammals: ERa and ERb are both nuclear receptors, whereas the G-protein coupled estrogen receptor 1 (GPER1 or GPR30) is a membrane ER (Prossnitz and Barton 2009; Thomas et al. 2005; Watson et al. 2011). ERa and ERb, which are encoded by the genes ESR1 and ESR2, respectively, are type 1 nuclear receptors normally located in the cytoplasm of responsive cells. Inactive ERs (not bound by ligand) are usually singly bound to corepressor complexes. Binding of ligand, notably the natural ligand 17b-estradiol (E2) and metabolites, induces dissociation of these complexes, dimerization (homo or hetero), and nuclear translocation, where the dimers bind to ER response elements upstream of responsive genes and alter gene expression (Shupnik 2002; Hewitt and Korach 2003; Nadal et al. 2005; Nilsson and Koehler 2005). In addition to transcriptional regulation, GPERs can activate mitogen-

activated protein kinases, increase intracellular cyclic adenosine monophosphate concentrations, and trigger calcium release from intracellular stores (Prossnitz and Barton 2009).

ERa and ERb have both been identified in alligator tissues (Katsu et al. 2004; Vonier et al. 1997). Studies indicate that estrogenic EDCs and endogenous E2 exhibit competitive binding for alligator ERs, suggesting that xenoestrogens may exert some of their deleterious effects through ER binding (Guillette et al. 2002; Rider et al. 2010; Vonier et al. 1996). Interestingly, there are noted differences in sensitivity between human and alligator ERs, with alligator ERs having a greater sensitivity to select EDCs (Rider et al. 2010). Thus, it is reasonable to predict that this increased ER sensitivity may be associated with enhanced abnormal physiological changes observed in alligators from EDC contaminated waters and thus warrants monitoring as a biomarker for xenoestrogen exposure.

Environmental estrogens, depending on concentration and frequency of exposure (Burnham et al. 2003; Kondo et al. 2004), could alter immune function through ER binding. Of note, ERs are expressed in most mammalian leukocytes, including cells of the myeloid and lymphoid lineage, suggesting that xenoestrogens can potentially modulate leukocyte development and function. Mammalian CD4+ and CD8+ T cells, B cells, and natural killer cells express ERa and ERb (Pierdominici et al. 2010). Expression has also been documented in macrophages, microglia, neutrophils and other granulocytes, and dendritic cells (Kovats and Carreras 2008; Molero et al. 2002; Seillet et al. 2012; Stygar et al. 2006; Turgeon et al. 2006). Furthermore, ERs have been identified in avian immune organs and leukocytes (Katayama et al. 2012; Shin et al.

2008), the closest extant crocodilian relative. Based on the previously mentioned studies, leukocytes are sensitive to estrogenic compounds (i.e., xenoestrogens) mediated through ERs. Because ERs have been characterized in alligators and are reported to be highly responsive to estrogen-mediated signaling, they are likely quite sensitive to xenoestrogens. Therefore, we can postulate that exposure to xenoestrogens, along with other EDCs (e.g., Muller et al. 2005), may modulate alligator leukocyte function through ER–leukocyte interaction and thus alter alligator immune function.

Estrogen-Induced Immune Pathology

Histopathology studies examining the impact of EDCs in crocodilians are few (refer to Table 3.3 for comparative studies). However, those few studies that do exist have suggested that exogenous exposure to environmental contaminants, such as EDCs, appear to damage immune lymphoid tissue (Rooney 1998; Rooney et al. 2003). In mammals, paradoxical findings illustrate that estrogenic exposure may differentially affect the immune system in distinct ways: stimulatory in some aspects, suppressive in others. Furthermore, xenoestrogens seem to affect various cell types in unique ways (Bouman et al. 2005; Carlsten et al. 1992; Chao et al. 2000; Islander et al. 2003). Although these distinctive outcomes may be due to the lineage affected, the time period of administration, or some other phenomena, the general consensus identifies estrogens as immunostimulators and androgens as immunosuppressors (Bouman et al. 2005; Islander et al. 2003; Nalbandian and Kovats 2005). In fact, human females compared with males at similar ages produce stronger humoral- and cell-mediated immune responses and are more resistant to infection but are more susceptible to autoimmune disorders, including

lupus, multiple sclerosis, celiac disease, and rheumatoid arthritis (Bouman et al. 2005; Gameiro et al. 2010; Nalbandian and Kovats 2005; Nussinovitch and Shoenfeld 2011; Tanriverdi et al. 2003). Estrogen-induced immunostimulation has been noted in a number of studies and is characterized by enhanced immune function associated with higher levels of estrogen (Karpuzoglu et al. 2007; Messingham et al. 2001; Rettew et al. 2009; Yoshino et al. 2003). Ironically, increased immunocompetence may disturb normal homeostasis and increase disease susceptibility (Cutolo et al. 2005, 2006; Komi et al. 2001; Kovats and Carreras 2008). For instance, estrogen-induced aberrant inflammation has been implicated in a number of immune-mediated disorders (Karpuzoglu and Ahmed 2006; Narita et al. 2007; Straub 2007). Contrasting with this stimulatory role, some mammalian studies suggest estrogens may have suppressive effects on the immune system and trigger immunodeficiency (Bouman et al. 2005; Dean et al. 1986; Fanti et al. 2003; Janis et al. 2004; Pung et al. 1985; Yellayi et al. 2003).

Estrogen-induced immunopathology has also been documented in avian species. With numerous homologies indicating common ancestry, birds are the closest extant relatives to crocodilians (Meyer and Zardoya 2003) and as such, a logical hypothesis might suggest that crocodilians may also demonstrate susceptibility to aberrant immune function triggered by estrogenic exposure. Birds are a mainstay in the alligator diet; therefore, ingestion of EDC-contaminated birds may cause dysfunction through accumulation and trophic magnification (Elsey et al. 2004). Immunopathologic EDC magnification has been observed in starlings (*Sturnus vulgaris*) with individuals displaying decreased T cell-mediated immune function after ingestion of diets contaminated with estrogenic EDCs (Markman et al. 2011). Japanese quail showed

similar results after estradiol (E2) exposure as shown by altered bursa of Fabricius development (Quinn et al. 2009). However, female chickens (*Gallus domesticus*), treated with growth- promoting diethylstilbestrol (DES), a nonsteroidal estrogen, had decreased antibody concentrations (Barua et al. 2000). However, DES treatment increased levels of immunocompetent cells (antigen-presenting cells, B cells, and T cells) in chickens (Zheng and Yoshimura 2001).

Nonavian reptiles, including lizards and turtles, demonstrate immune-related pathologies after estrogen administration. In red-eared sliders (*Trachemys scripta*), exposure to estrogenic polychlorinated biphenyls (PCBs) resulted in sex reversal, with females produced at male- incubating temperatures (Bergeron et al. 1994; Crews et al. 1995). Furthermore, PCB exposure altered immune function in loggerhead sea turtles (*Caretta caretta*) by suppressing innate functions while enhancing adaptive functions (Keller et al. 2006). Similar findings of immune dysregulation were observed in lizards. For example, E2 exposure induced thymic atrophy and inhibited thymocyte proliferation in *Hemidactylus flaviviridis*, and injection of 17a-ethinylestradiol, an environmental estrogen often present in sewage-treatment plant effluent, decreased peripheral blood leukocyte and total splenocyte levels in *Sceloporus occidentalis* (Burnham et al. 2003; Harerama- das and Rai 2006).

Alligators inhabit an aquatic environment, thus aquatic exposure to estrogenic compounds through ingestion of water or aquatic organisms may increase susceptibility to immune dysfunction. Fish and mollusks are essential dietary components with invertebrate nutrition predominating in hatchling and juvenile alligators (Gabrey 2010; Platt et al. 1990). Numerous studies have shown immune dysfunction after estrogenic

exposure; thus, predation of aforementioned organisms may represent possible routes for magnification of estrogens by way of dietary assimilation (Cabas et al. 2012; Canesi et al. 2005; Gauthier- Clerc et al. 2006; Milston et al. 2003). For example, zebrafish exhibit increased mRNA levels of cytokines, nitric oxide synthase, and genes associated with oxidative stress after exposure to estrogenic EDCs (Jin et al. 2010). In addition, alterations in leukocyte proliferation and phagocytosis have been observed after exposure to estrogenic compounds in rainbow trout (*Oncorhyncus mykiss*) and tilapia (*Oreochromis niloticus* X *O. aureus*), respectively (Law et al. 2001; Shelley et al. 2012). Phagocytic alterations are also observed in bivalves with low concentrations of E2 stimulating phagocytosis and high concentrations suppressing phagocytosis in mussels (*Mytilus galloprovincialis*) (Canesi et al. 2006).

Conclusion

The role of EDCs, in particular, environmental estrogens, and their effects on immune function in alligators are currently understudied with most studies mainly investigating adverse effects in mammals, non-crocodilian reptiles, fish, and invertebrates. Physiologically, sex hormones modulate immune function with both estrogens and androgens impacting immunocompetence. Not surprisingly, estrogenic EDCs, which may structurally resemble estrogens or act similarly, disrupt normal immune homeostasis (Ahmed 2000). This disruption may be two-pronged: estrogenic EDCs may induce autoimmune susceptibility by way of an overstimulated immune response, while at the same time promoting infection and disease development through specific immune deficiencies.

Crocodilians potentially can function as effective environmental sentinels for EDC exposure: They are apex predators and long-lived, thus allowing for the bioaccumulation of chemicals, metabolites, and toxins over an extended period of time (Milnes and Guillette 2008; Yoshikane et al. 2006). Reproductive and developmental abnormalities are well characterized in EDC-exposed crocodilians, although estrogenic action is not solely restricted to reproductive tissues. It has been shown that exposure to estrogenic EDCs, along with heavy metals, may induce alterations in deposition of maternally derived steroids and consequently may precipitate reproductive abnormalities in embryos by way of maternal transfer (Hamlin et al. 2010). Because alligators are top-trophic consumers, this status may allow for the biomagnification of these compounds and increased deposition in older, mature organisms (Fossi et al. 2000, 2006; Heinz et al. 1991; Roe et al. 2004). Thus, prolonged exposure would likely result in increased body burdens in both paternal and maternal tissues. This may in turn affect spermatogenesis in males, alter gonadal morphology and function, directly affect embryonic development, decrease overall fitness, and likely affect immune function through estrogenic accumulation throughout life history (Guillette et al. 2000). Increased ER activation may induce hyperactive responsiveness manifesting in increased disease susceptibility or direct suppression of specific immune parameters.

Presently, the effects of environmental estrogens on crocodilian immune functions are not well characterized, although studies on the Lake Apopka alligators clearly indicate that these animals display an altered physiology, which would likely affect their resistance to pathogens and disease. In particular, preliminary studies in alligators indicate that environmental estrogen exposure affects immune function with

the spleen and thymus as possible targets (Rooney 1998; Rooney et al. 2003). Because alligator immune function is in part under hormonal control with temperatures influencing release (Zapata et al. 1992), any perturbations in hormone concentrations may affect immunity. To summarize, sufficient evidence exists linking the sensitivity of alligators to environmental endocrine-active contaminants. New and more detailed studies evaluating the effects of these compounds on alligator physiology, including a more in-depth assessment of immune function in these top-trophic carnivores, is warranted to assess whether they are effective environmental sentinels for EDC exposure.

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Tables

Table 3.1: Immune organs and associated tissues of amniotic vertebrates.

Lymphoid-associated tissue	**Non-archosaurian reptiles[a,b,e]**	**Crocodilians[a,b,c,e,f]**	**Birds[d]**	**Mammals[d,e]**
Gut-associated lymphoid tissue	+	+	+	+
Thymus	+	+	+	+
Spleen	+	+	+	+
Lymph Nodes	-	-	+	+
Bursa of Fabricius	-	-	+	-
Bone Marrow	+	+	+	+
Germinal Center Formation	-	-	+	+

[a]Leceta and Zapata 1985; [b]Zapata et al. 1992; [c]Tanaka and Elsey 1997; [d]Fairbrother et al. 2002; [e]Zimmerman et al. 2010b; [f]Finger and Isberg, 2012

Table 3.2 Leukocytes of amniotic vertebrates.

Cell type	**Non-archosaurian reptiles[d,e]**	**Crocodilians[a,b,d]**	**Birds[c]**	**Mammals[d,e]**
Heterophils	+	+	+	-
Azurophils	+	-	-	-
Eosinophils	+	+	+	+
Basophils	+	+	+	+
Neutrophils	-	-	-	+
Monocytes	+	+	+	+
Lymphocytes	+	+	+	+

[a]Cuchens and Clem 1979; [b]Mateo et al. 1984; [c]Harmon 1998; [d]Sykes and Klaphake 2008; [e]Zimmerman et al. 2010b

Table 3.3 Effects of natural and environmental estrogens on wildlife immune response.

Chemical(s)	Species	Effects	Reference
17α-ethynylestradiol	*Sparus aurata*	Decreased *in vivo* production of IL-1β; increased *in vitro* expression of inflammatory cytokines IL-6, IL-1β, and TNF-α	Cabas et al. 2012
17β-estradiol	*Coturnix japonica*	Decreased age-associted atrophy in the bursa of Fabricius following *in ovo* injection	Quinn et al. 2009
17β-estradiol	*Hemidactylus flaviviridis*	Dose dependent decrease in thymocyte proliferation in presence of T cell mitogen Con-A	Hareramadas and Rai 2006
17β-estradiol	*Homo sapiens*	Increased expression of neuronal nitric oxide synthase in neutrophils	Molero et al. 2002
17β-estradiol	*Homo sapiens*	*In vitro* monocyte/macrophage cell line exhibited increased expression of NF-κB, subsequent increased NF-κB DNA binding, and decreased apoptosis	Cutolo et al. 2005
17β-estradiol	*Mus musculus*	Increased transcription of TLR2, TNF-α, IL-12 following LPS injection	Soucy et al. 2005
17β-estradiol	*Mus musculus*	Increased TLR4 and CD14 expression in macrophages	Rettew et al. 2009
17β-estradiol (E2)	*Mya arenaria*	Inhibition of hemocyte phagocytosis at concentrations of 10 and 20 nmol E2	Gauthier-Clerc et al. 2006
17β-estradiol (E2)	*Mytilus galloprovincialis*	Increased hemocyte phagocytosis at 5 and 25 nM E2; decreased phagocytosis at 50 nM E2	Canesi et al. 2006
17β-estradiol, dioctyl phthalate, dibutyl phthalate, bisphenol A	*Sturnis vulgaris*	Lowered response of wing web swelling following injection of PHA	Markman et al. 2011
4,4'-DDE	*Caretta caretta*	Increased concentration of chemical negatively correlated with lysozyme activity	Keller et al. 2006
Dicofol, DDT and metabolites	*Alligator mississippiensis*	Decreased thymic ratio (medulla:cortex); decreased size of malpighian bodies and lymphocyte sheaths in spleen	Rooney et al. 2003

Diethylstilbestrol	*Mus musculus*	Decreased production of IL-2 and subsequent increased infection with *Listeria monocytogenes*	Pung et al. 1985
Genistein	*Mus musculus*	Decreased numbers of CD4+ and CD8+ T cells; decreased delayed-type hypersensitivy response following injection with exogenous hapten	Yellayi et al. 2003
Organochlorines (OC)	*Caretta caretta*	Positive corelation between WBC cell counts and heterophil:lymphocyte ratios to OC concentrations	Keller et al. 2004
Organochlorines (OC)	*Sterna caspia*	Strong negative correlation in PHA response (T cell mitogen) and percentage of blood monocytes to plasma PCB levels; Positive corelation in antibody titers to SRBCs and plasma PCB levels	Grasman and Fox 2001

IL interleukin, *TFF* tumor-necrosis factor, *TLR* toll-like receptor, *SRBC* sheep red blood cells

Figures

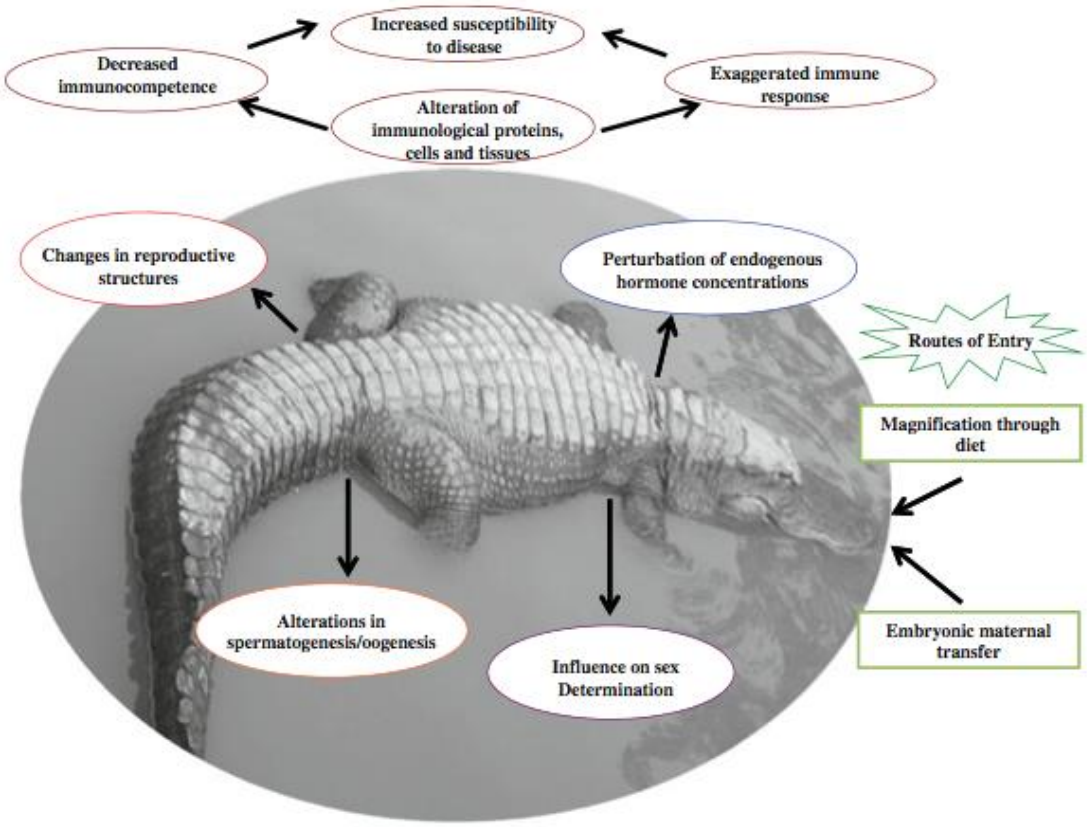

Figure 3.1 The influence of environmental estrogens on American alligator physiology. Estrogenic chemicals affect various aspects of alligator physiology through trophic magnification and embryonic maternal transfer (routes of entry), including changes in TSD, endogenous hormone concentrations, reproductive abnormalities, and some aspects of immune function. Thorough elucidation of the immunological effects of estrogenic EDCs on alligator immunity is lacking

CHAPTER 4

USING PHYTOHAEMAGGLUTININ TO DETERMINE IMMUNE RESPONSIVENESS IN SALTWATER CROCODILES, *CROCODYLUS POROSUS* [3]

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[3] Finger, J.W. Jr., A.L. Adams, P.C. Thomson, C.M. Shilton, G.P. Brown, C. Moran, L.G. Miles, T.C. Glenn, and S.R. Isberg. 2013. *Australian Journal of Zoology*. 61:301-311. Reprinted here with permission of the Publisher

Abstract

Immune responsiveness, the ability of an organism to effectively respond immunologically following antigenic exposure, is an essential component of life history, as organisms require effective immune functionality in order to grow, survive and reproduce. However immune status is also associated with concomitant trade-offs in these physiological functions. Herein we demonstrate the validation of phytohaemagglutinin (PHA) injection in saltwater crocodiles, *Crocodylus porosus*, to assess cellular immune responsiveness. Following injection of 2 mg mL^{-1} PHA into the hind toe webbing, we observed a peak swelling response 12 hours post injection, with PHA inducing increased thickness compared to PBS injected webs ($F_{5,518}$=145.13, p<0.001). Subsequent injections increased responsiveness relative to the primary injection response ($F_{5,290}$=2.92, p=0.029) suggesting PHA exposure induced immunological memory, a tenet of acquired immunity. Histological examination revealed PHA-injected toe webs displayed increased numbers of leukocytes (granulocytes, macrophages, and lymphocytes) relative to PBS injected webs, with peak leukocytic infiltrate observed 12 hours post injection. We suggest the use of PHA injection in crocodilians as a measure of cellular immune responsiveness in agricultural (production and animal welfare), ecological, and toxicological contexts.

Introduction

Of paramount importance in the life history of an organism is the capacity to mount an efficacious immune response following antigenic exposure (Vinkler and Albrect 2011; Zuk and Stoehr 2002). This capacity, known as immune responsiveness (Vinkler and Albrect 2011), is often associated with concomitant tradeoffs in other

physiological parameters, with augmented immunity negatively affecting growth, reproduction, and survival (Bascuñán-García et al. 2010; Gutierrez et al. 2013; Martin et al. 2006; Martin et al. 2008; McCallum and Trauth 2007; Ruiz et al. 2011; Zuk and Johnsen 1998). Due to these interdependent effects, determination of immune status is important in a number of broad disciplines. An understanding of innate and acquired immune functions is necessary in immunoecological studies to determine how immune status may affect fecundity and other physiological parameters (Brown et al. 2011; Brzek and Konarzewski 2007; Demas et al. 2011; Duffy and Ball 2002; Smits et al. 1999; Tschirren et al. 2007). In toxicological studies, determination of immune status following contaminant exposure is required to understand how exposure may detrimentally impact immune function (Grasman 2002; Keller et al. 2006; Markman et al. 2011; Smits et al. 1999; Smits and Williams 1999), possibly culminating in increased disease susceptibility or exaggerated responsiveness (hypersensitivity) (Fairbrother et al. 2004; Youssef et al. 1996). Similarly in animal production systems, the understanding of factors affecting immunity such as microbial infection or other stressors (Shini et al. 2008), are imperative as such costs restrict resource allocation for increased growth (Bonato et al. 2009; Chen et al. 2007; Fair et al. 1999;) and reproduction (Boughton et al. 2007). Indeed immune responsiveness may also provide information to develop animal welfare guidelines (Caipang et al. 2008; Hanlon et al. 1994; Segner et al. 2012). Thus, techniques to ascertain immune responsiveness are necessary amongst a variety of scientific disciplines.

Techniques to determine immune functionality generally fall under two general approaches: immunomonitoring or immunochallenge, with the former often highly

criticised for lack of standardization and the latter allowing for treatment standardization among individuals and direct quantification of response following administration (Norris and Evans 2000). One such immunochallenge technique involves intradermal injection of phytohaemagglutinin (PHA), a non-pathogenic, antigenic-lectin derived from the red kidney bean (*Phaseolus vulgaris*), with repeated measurements of the subsequent swelling response at the injection site enabling evaluation of organism's immune response to antigenic stimulation (Guitierrez et al. 2013; Vinkler and Albrect 2011). PHA was first utilised as a measure of cell-mediated immunity, with *in vitro* exposure inducing augmented T-lymphocyte mitogenesis in human leukocytes (Morgan et al. 1976; Nowell 1960). Following *in vivo* injection, PHA acts as a polyclonal antigen inducing enhanced endothelial permeability, triggering edema and leukocyte migration out of blood vessels into tissues (Brown et al. 2011; Turmelle et al. 2010). Initial injections trigger a primary (innate, non-specific) immune response, with infiltration of a whole host of innate cells including granulocytes and macrophages (Kennedy and Nager 2006; Martin et al. 2006), along with lymphocytes, the mediators of acquired (adaptive, specific) immunity. During a normal microbial infection, these innate cells activate lymphocytes, which differentiate into effector and memory cells, the former of which aid in elimination of antigen and the latter of which are important in repeated exposures (Abbas et al. 2010). Compared to primary injections, subsequent injections of PHA exhibit an increased responsiveness (more swelling) showing PHA can induce immunological memory (Brown et al. 2011; Tella et al. 2008), a tenet of acquired immunity. Thus, PHA stimulation may elucidate both the primary immune response

(primary injection) and acquired immune response (repeated injections) in an individual (Demas et al. 2011).

The impetus for this study on saltwater crocodiles was two-fold. Firstly, as with other crocodilians, saltwater crocodiles occupy top trophic status in their semi-aquatic environment. Therefore, crocodilians are important environmental indicators for events such as toxicant exposure (Milnes and Guillette 2008). Secondly, saltwater crocodile skin is highly sought by the international skin trade, and consequently farming crocodiles for their skin has become a sustainable economic enterprise in Northern Australia with proven conservation benefits (references within Fukuda et al. 2011). In this respect, an understanding of both the innate and adaptive immune system would be beneficial to alleviate the on-farm risk of disease, increase survival (Isberg et al. 2006; Isberg et al. 2009), enhance growth (Isberg et al. 2005) and, furthermore, develop animal welfare guidelines. Finally, crocodilian immune function is very poorly characterised (Finger and Isberg 2012), so the development of inexpensive and easy techniques will provide a foundation for further research.

Before PHA can be used to assess cellular immune responsiveness in a species, the appropriate concentration and volume for injection must be determined, the time points for repeated measurements defined and qualified according to the infiltration of different immunological cell types, and an immunological memory demonstrated following re-injection. Herein we achieve these objectives on juvenile saltwater crocodiles (*Crocodylus porosus*), using an experimental design similar to that described by Brown et al. (2011) for cane toads (*Rhinella marina*).

Methods and Materials

Study Species

Three month old saltwater crocodiles from Darwin Crocodile Farm, Noonamah, Northern Territory, Australia were used in this study. The animals were housed in pens (35 in each; pen dimensions: 116.5 cm wide and 209.5 cm long) with the water temperature maintained at 32°C (± 1°C). The animals were fed in excess five days per week in the evenings and the pens were cleaned the following morning with a chlorine-based detergent. For sampling, crocodiles were randomly selected and injected as described below, then placed in individual containers and placed within a dark humidified (90-100%) incubator at a constant temperature of 32°C (± 0.5°C). As our study coincided with normal non-feeding periods, crocodiles were not provided with food during the sampling period. Upon completion, animals were returned to their respective pens.

Experimental Protocol

PHA (PHA-P #L8754; Sigma-Aldrich, St. Louis, MO, USA) was aseptically dissolved in sterile phosphate buffered saline (PBS) and 0.02 mL was injected into the toe web between the first and second hind digits using a 0.3 mL syringe with a 29-gauge needle. Sterile PBS was used as the control and 0.02 mL was injected into the opposing toe web of each animal. PBS was autoclaved and subsequently aliquoted into 1.5 mL tubes and stored at 4°C prior to and following administration. The volume to inject was standardised at 0.02 mL as this amount was readily observed subcutaneously in the toe web upon successful injection, the whole amount could be injected without any fluid leaking out the point of injection, and the fluid quickly dissipated within a short time post

injection (Brown et al. 2011). One person (JWF) performed all the injections to standardise the procedure. Prior to administration, toe webs were swabbed with alcohol in an attempt to prevent pathogenic entry upon injection.

Toe web thickness was measured prior to (0 hr) and after injection (6, 12, 24, 48 and 72 hr) using a dial thickness gauge (Peacock G-1A; Ozaki Manufacturing Ltd., Japan). Three measurements were taken in quick succession at each time point, allowing an initial contact of less than 2 seconds to determine swelling thickness. Gauge contact pushed fluid out of the immediate area, thus quick measurements were of paramount importance to ascertain true thickness (Brown et al. 2011). Measurements were performed by one person (JWF) to standardise time of contact with swelled skin. The three measurements were averaged to determine the average swelling of each individual at each respective time points. Head length of each individual was measured using digital calipers (nearest 0.01 mm) from snout tip to the median posterior of the cranial platform as a measure of overall length (Isberg et al. 2005; Webb and Messel 1978).

Dose Effects

An appropriate concentration was determined by injecting 0.02 mL of 1, 2, or 5 mg mL^{-1} of PHA and examining the effects of swelling. A total of 24 animals were injected, with eight animals randomly allocated into each dosage group. Following statistical analysis, 2 mg mL^{-1} was selected as the concentration of PHA used in all subsequent experiments.

Temporal Effects and Histological Verification

Whilst acknowledging that the swelling response is a combination of innate and cell-mediated immune activation that needs to be further qualified at various time points

(see below), the next objective was to determine the temporal response after injection. As such, 60 animals (together with 8 injected with 2 mg mL^{-1} from the previous experiment for a total of 68) were injected with 0.02 ml PHA and PBS (control) in the left and right toe webs, respectively. The toe web thickness was measured before injection (0 hr) and then at 6, 12, 24, 48 and 72 hr post-injection. Of these 60 animals, 30 were also used in the histological examination of cell-type (five at each time point) so the swelling response of 60, 55, 50, 45, 40 and 35 animals were available for analysis at each time point respectively.

To qualify the cellular basis of the swelling response, toe webs were histologically examined as previous studies have illustrated that PHA increases T lymphocyte migration, a component of acquired immunity (Brown et al. 2011; Tella et al. 2008), but other cell types are also attracted to the site of injection including innate cells such as macrophages and granulocytes (Martin et al. 2006). Three millimeter diameter biopsies, from both the PHA and PBS (control) toe webs, juxtaposed to the site of injection were taken from five animals at each time point (0, 6, 12, 24, 48, and 72 hr) and stored in 10% buffered formalin. Biopsies were processed for histological examination by embedding in paraffin, sectioning at 4 μm and staining with hematoxylin and eosin (H & E). As histological examination is subjective, to minimise bias, biopsies were examined independently by two individuals (JWF and ALA) unaware of the time point the biopsy was taken. Macrophages, granulocytes, and lymphocytes were quantified using a light microscope at 400× magnification following standard staining characteristics attributed to each cell type (Canfield 1985; Glassman et al. 1981; Zayas et al. 2011). Biopsies were divided into adjacent fields (Brown et al. 2011), with cell types enumerated in each field

and subsequently summed, providing a total count of all leukocytes in the biopsy. Total counts by JWF and ALA at respective time points were averaged. Lymphocyte type cannot be distinguished with H&E under light microscopy, therefore the counts reflect total number of lymphocytes and are not restricted to T cells (Brown et al. 2011). Similarly, granulocytes with eosinophilic granules were not differentiated but instead reflect the total sum of heterophils and eosinophils.

Temporal Replication

As well as determining the temporal response, one of the tenets of cell-mediated immunity is immunological memory. That is, subsequent injections of PHA should induce an increased swelling response when compared with the initial injection (Brown et al. 2011; Tella et al. 2008). To determine the role of memory upon subsequent injection with PHA, the 30 animals not used in the histological examination were re-injected. The second injection occurred 21 days after the initial injection when web thickness was assumed to have returned to pre-injection thickness. The second PHA injection was administered into the opposite toe web of the initial injection (i.e. PHA in right toe web first and into left toe web second). Following primary injections, crocodiles were fitted with individual tags (National Band and Tag Co., USA) on their first single-row scute (Isberg et al. 2004) to enable easy identification for the subsequent injections.

Statistical Analysis

Analyses were conducted using restricted maximum likelihood (REML) in GenStat (Version 14, VSN International) with web thickness at their respective time points serving as the response variable. In all analyses, Animal was included as a random effect to account for paired observations between PHA- and PBS-injected toe webs.

Animal size, as indicated by head length (HL; covariate), time, treatment and a time × treatment interaction were included as fixed effects in all models. The term Replication was also included for the temporal replication analysis along with all possible interactions. A serial correlation structure was accounted for within the model structure for each analysis. Following histological examination, enumerated leukocytes (granulocyte, macrophage, or lymphocyte) were analysed using a generalised linear mixed model (GLMM) with a Poisson distribution with cell type count used as the response variate and time, treatment and their interaction used as fixed effects. Animal was again included as a random effect to account for paired observations. Reported estimates are presented as back-transformed means.

Results

Effects of Body Size on Web Thickness

Prior to injection, the average thickness of left and right toe webs were similar ($F_{1,82}$=0.29, p=0.593). However, there was a significant effect of crocodile size (HL) on initial toe webbing thickness (1.11×10^{-2} mm mm^{-1}; SE=9.76×10^{-4}; $F_{1,81}$=129.27, p<0.001) (Figure 4.1), although one animal was removed from the analysis as its head length (HL) was an extreme outlier. Consequently, HL was used as a covariate in subsequent analyses whenever significant.

Dose Effects

There was a significant time by dose interaction ($F_{15,22}$=7.12, p<0.001). Using the least significant difference (5% LSD), the 1 mg mL^{-1} dose was significantly different from the control (PBS) at 24 and 48 hours only, whereas the 2 and 5 mg mL^{-1} were significantly different at all time points with the exception of 2 mg mL^{-1} at 72 hours

(Figure 4.2). As a result, we chose to conduct all further examinations using the lower dose of 2 mg mL^{-1} (Smits and Williams 1999). Animal size also had a significant effect on swelling ($F_{1,20}$=11.03, p=0.003).

Temporal Profile of PHA-Induced Swelling

To clarify the time effect on the swelling response, 60 additional crocodiles were injected allowing for 68 crocodiles to be examined when combined with the original eight from the dose effect trial. However, of these additional 60 crocodiles, 30 (five at each time point) were randomly chosen for biopsies and could not be re-measured after the biopsy was taken. Therefore, there were totals of 68, 63, 58, 53, 48 and 43 crocodiles (i.e. the decrease reflecting animals unavailable for measurement due to biopsy) available for measurement of toe web thickness at each time point. PHA injected toe webs exhibited amplified swelling compared to PBS injected webs although this varied over time (time×treatment interaction $F_{5,55}$=75.44, p<0.001), with PHA-induced swelling significantly increased (5% LSD) at 6, 12, 24, 48, and 72 hours (Figure 4.3). Peak swelling response was observed 12 hours post-injection for both PHA- and PBS-injected toe webs, with an increase in thickness of 66.7% (0.45 to 0.75 mm) and 15.6% (0.45 to 0.52 mm), respectively (Figure 4.3). As expected, larger crocodiles showed a greater response (HL; $F_{1,36}$=90.75, p<0.001).

Temporal Replication

As PHA is purported to stimulate the cell-mediated response of the acquired immune system, we would expect increasing responsiveness upon subsequent stimulation with the same antigen. Secondary administration had a significant effect on swelling response (replication×time×treatment $F_{5,693}$=2.26, p=0.047). Secondary injection of PHA

significantly induced more swelling than primary injection at all-time points except 6 hours (rep×time×treatment 5% LSD) (Figure 4.4). Interestingly, secondary injection of PBS also induced more swelling than primary injection, with significant increases observed at 0, 24, and 48 hours (rep×time×treatment 5% LSD). The results observed at 0 hours with both PBS and PHA may suggest the time period of 21 days between injections was not sufficient to reduce swelling back to pre-injection levels, as PHA injection may have long lasting effects on the immune system (see Sarv and Horack 2009). However, these differences may also reflect a natural increase in thickness between the two injection periods, as farmed saltwater crocodiles experience pronounced growth during the first few years of life (Isberg et al. 2004; Isberg et al. 2005). Secondary responsiveness was not affected by animal size ($F_{1,32}$=3.85, p=0.058).

Histological Examination

Examination revealed a significant infiltration of granulocytes ($F_{5,25}$=3.00, p=0.03), lymphocytes ($F_{5,26}$=3.34, p=0.018), and macrophages ($F_{5,24}$=5.87, p=0.001) at all time points after injection (0 hr) (approximate 5% LSD = 2 × SED) in PHA-injected webs compared to control webs. Peak infiltration of macrophages (Figure 4.5A) and lymphocytes (Figure 4.5B) occurred at 12 hours post-injection (Figure 4.6 and 4.7), whereas peak granulocytes infiltration (Figure 4.5C) occurred at 6 hours. Animal size had no effect on cellular infiltration (granulocytes $F_{1,14}$=0.01, p=0.937; lymphocytes $F_{5,25}$=0.60, p=0.447; macrophages $F_{1,19}$=0.83, p=0.374).

Discussion

Herein we have validated the use of PHA and suggested its use to measure immune responsiveness in the saltwater crocodile, *Crocodylus porosus*. After injection,

the augmented swelling peaked at 12 hours with larger responses observed in larger animals. Histological examination revealed primary injection initially stimulated innate cellular infiltration by first recruiting granulocytes (peaking at 6 hours), followed by peak numbers of macrophages and lymphocytes at 12 hours, however it is doubtful that these lymphocytes embody newly formed progeny of PHA-stimulated lymphocytes this soon after injection (Brown et al. 2011). However, subsequent administration revealed an enhanced swelling response, suggesting PHA induces an adaptive immune response in juvenile saltwater crocodiles. These results correspond with what others have observed in birds (Tella et al. 2008) and anurans (Brown et al. 2011), with subsequent injections of PHA increasing swelling response.

Analogous to our results, Zimmerman et al. (2010) observed a peak swelling response to PHA 12 hours post-injection in red-eared sliders, *Trachemys scripta* and Turmelle et al. (2010) observed peak swelling response and peak lymphocyte infiltration 12 hours post-injection in Brazilian free-tailed bats *Tardarida brasiliensis*. Within Aves, mixed results have been obtained with a number of studies showing peak swelling responses between 12 and 24 hours (Grasman 2002). By contrast, Martin et al. (2006) observed the peak swelling response at 48 hours post-injection in house sparrows (*Passer domesticus*), although the maximum lymphocyte and heterophil counts were detected after 6 and 12 hours, respectively. In ostriches (*Struthio camelus*), swelling at 6 hours post-injection was not significantly different from those at 24 hours (Bonato et al. 2009), albeit measurements were not taken at 12 hours. In cane toads, Brown et al. (2011) observed both peak lymphocytic infiltration and peak swelling 24 hours post-injection, although peak numbers of granulocytes and macrophages were observed 12 hours post-

injection, similar to our findings. Interestingly, the greatest swelling response in another anuran, the southern leopard frog (*Rana sphenocephala*), was observed 48 hours post-injection but no histological examinations were undertaken to quantify the cell type infiltration (Venesky et al. 2012). These examples show the lack of interspecific standardization in peak swelling response and leukocyte infiltration, with such high variability probably attributable to a number of factors. As such, direct comparisons in swelling response may prove unwise (see Matson et al. 2006), requiring independent validation for the study species of interest.

Whilst demonstrating the use of this technique as a measure of immune function in a crocodilian species, the response elicited may be affected by multiple biotic, such as diet or disease status (Tschirren et al. 2007; Venesky et al. 2012), and abiotic factors. As such, results obtained in this study using farmed saltwater crocodiles raised within temperature-controlled sheds may be different than using crocodiles obtained from wild habitats, from other farms with different management regimes, or at differing ages or seasons (Schwanz et al. 2011; Zimmerman et al. 2010). Efficacy of ectothermic immune response is dependent on temperature, and as such, seasonality may affect swelling response (Martin et al. 2008; Zapata et al. 1992; Zimmerman et al. 2010). Seasonality (and temperature) may also affect levels of endogenous sex steroids even in sexually immature crocodilians (Rooney et al. 2004) and, as sex steroids affect immune function (Lutton and Callard 2006), variation in response to PHA challenge may be observed depending on season of administration. Furthermore, animals in farming situations as opposed to natural environments may be exposed to different types of stressors (Elsey et al. 1990). Thus, differences in swelling response may arise due to corticosterone-

mediated immunosuppression (Martin et al. 2005). Indeed, saltwater crocodiles housed at higher stocking densities demonstrated elevated corticosterone, with subsequent increased perturbations in immune function and increased susceptibility to opportunistic infection (Turton et al. 1997). Moreover, circadian cycles of dark and light are also known to affect corticosterone secretion in crocodilians (Lance and Lauren 1984) and the crocodiles used herein were housed in temperature-controlled sheds which may restrict natural circadian rhythms, possibly affecting corticosterone levels and, consequently, swelling response.

Furthermore, it is important to emphasise that no one technique can serve as a quintessential immunological measure and instead PHA injection should be coupled with other immunological assays for better assessment of immune responsiveness (Demas et al. 2011; Norris and Evans 2000), as an individual's responsiveness to one antigen may not be indicative of its responsiveness to another. In fact, many studies incorporate measures of multiple immune parameters to aid in elucidation of immunity in an individual (e.g. Fair et al. 1999; Schwanz et al. 2011). When interpreting results from PHA injection, cautionary analysis must be employed (Martin et al. 2006). For example, as primary injections are composed of both innate and adaptive components, an enhanced swelling response to primary injection may not be indicative of an enhanced cell-mediated (adaptive) response (Martin et al. 2006). In fact, subsequent injections are necessary for evaluation of the adaptive (acquired) response (Demas et al. 2011), as the adaptive immune response changes (adapts) with repeated exposures to same antigen (Abbas et al. 2010). Furthermore, higher quality individuals may not necessarily develop a greater response compared to lower quality individuals (Vinkler et al. 2012). The type

of PHA lectin utilised in experimentation may also influence the response generated and thus, confound results (see Vinkler et al. 2010). Therefore it is important to be aware of these and other confounding variables when conducting experimentation using PHA injection.

PHA administration has been utilised in many disciplines to understand different variables affecting immunity, including immunotoxicological (Fairbrother et al. 2004; Grasman 2002; Markman et al. 2011; Muller et al. 2005; Smits and Williams 1999), agricultural (Bonato et al. 2009; Boughton et al. 2007; Hernandez et al. 2005), and ecological studies (Brzek and Konarzewski 2007; Martin et al. 2006; Smits et al. 1999; Tschirren et al. 2007). Thus, we propose similar use to determine immune status in studies relating to crocodilians. Crocodilians are important components of their ecosystem, acting as top trophic carnivores, serving as sentinels of environmental quality (Milnes and Guillette 2008), and in some species, modifying ecosystems tor the benefit of other species (Craighead 1968; Magnusson and Taylor 1982). Furthermore, use of crocodilians is important in a number of countries, including Australia (Isberg et al. 2004), where leather products made from the skins provide employment and economic incentives for sustainable use. Thus, the utilization of a technique to measure immune responsiveness, such as PHA administration, in crocodilians may enable further investigation throughout a number of wide-ranging disciplines and provide greater understanding of the immune systems of these organisms.

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and ALA access to facilities for examining biopsies. This project was funded by a grant from the Rural Industries Research and Development Corporation, Australia. All experimental protocols were approved by the University of Sydney Animal Ethics committee (approval number: N00/5-2012/3/5729). We also thank the *Australian Journal of Zoology* and CSIRO Publishing for permission to use "Using phytohaemagglutinin to determine immune responsiveness in saltwater crocodiles, *Crocodylus porosus*" (*Australian Journal of Zoology* 61(4) 301-311 http://dx.doi.org/10.1071/ZO13041) in this dissertation. The other authors on this paper include Amanda, L. Adams, Peter C. Thomson, Cathy M. Shilton, Greg P. Brown, Christopher Moran, Lee G. Miles, Travis C. Glenn, and Sally R. Isberg. Paper can be accessed at the following website: http://www.publish.csiro.au/?paper=ZO13041 / http://dx.doi.org/10.1071/ZO13041.

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Figures

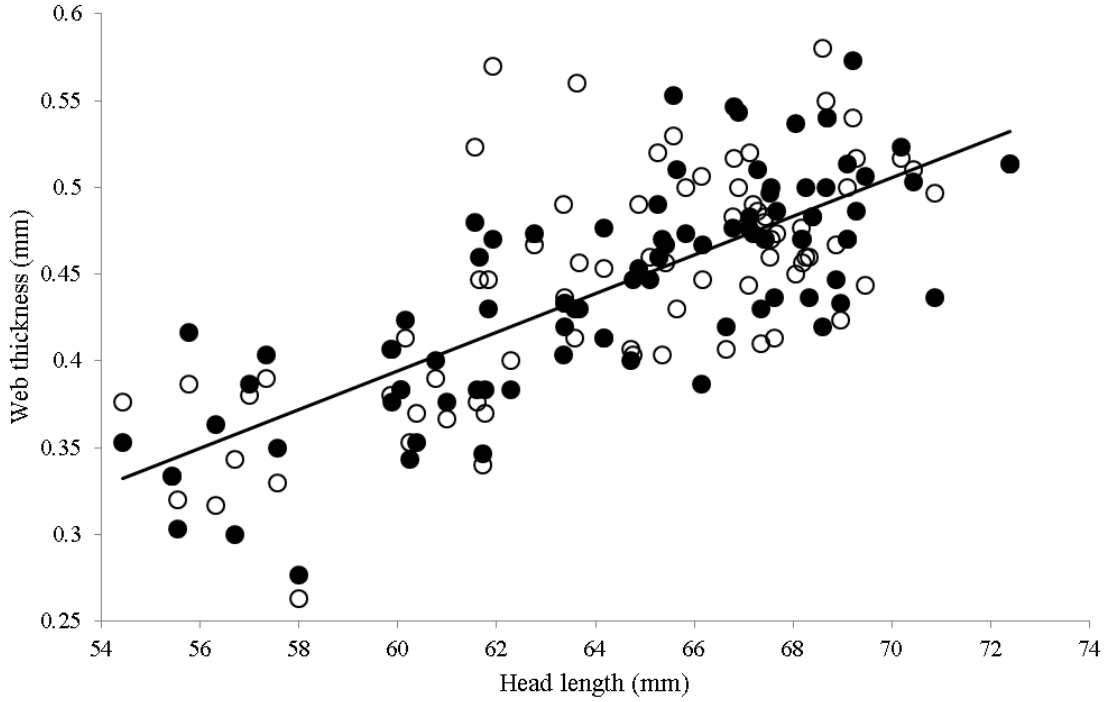

Figure 4.1 Relationship between crocodile size, as indicated by head length (HL, mm) and pre-treatment toe web thickness (mm).

Closed and open circles indicate the left and right toe web thicknesses, respectively.

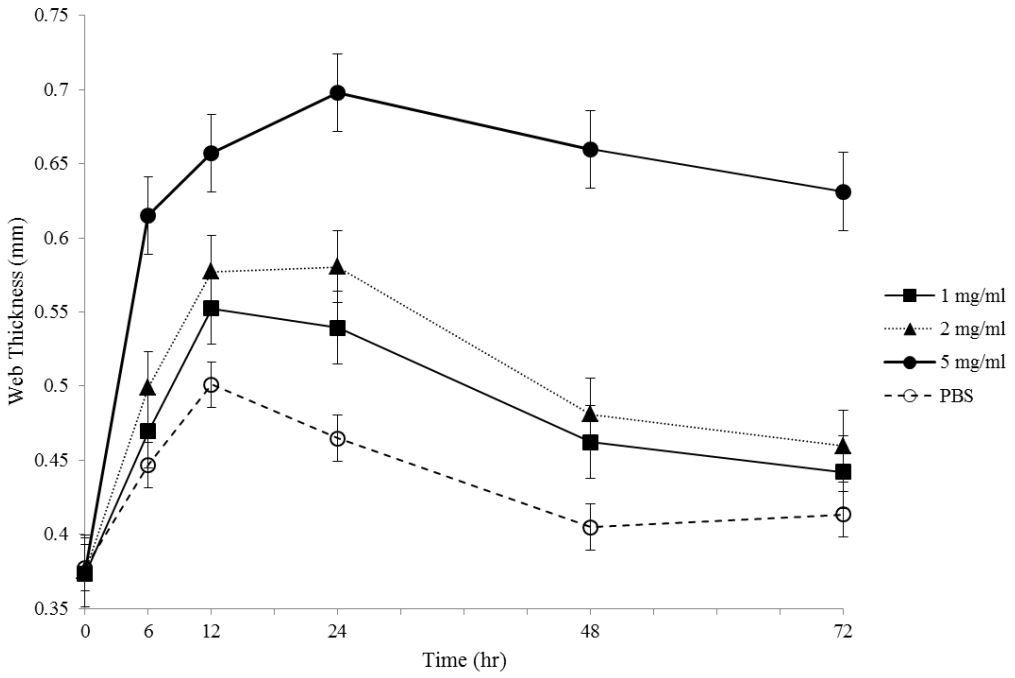

Figure 4.2 The effects of PBS (dashed black line, open circles) and 1 (squares), 2 (triangles), and 5 mg mL^{-1} (solid circles) PHA on average (±SE) swelling response (mm) over time (hours).

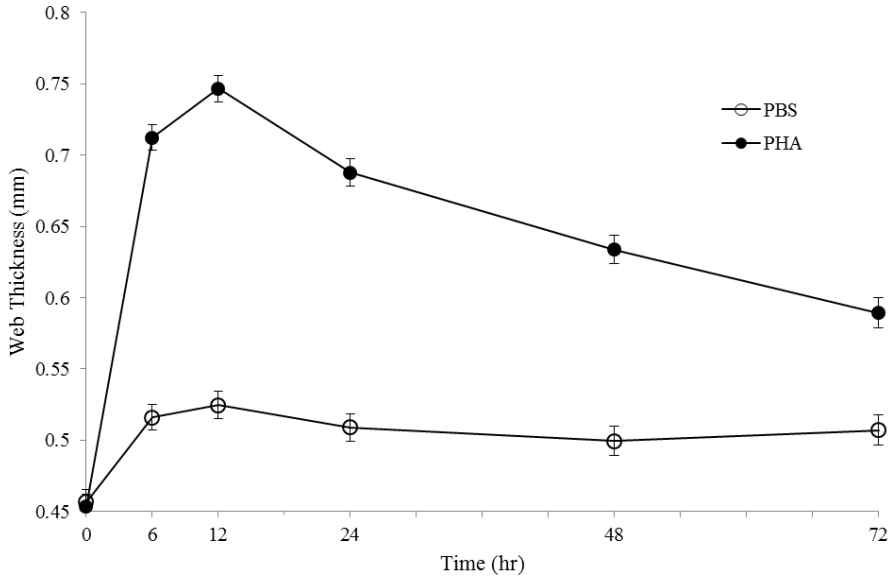

Figure 4.3 The effects of injection of PHA (solid circles) and PBS (dashed black line, open circles) on average (±SE) swelling response (mm) in juvenile saltwater crocodile toe webs over time (hours).

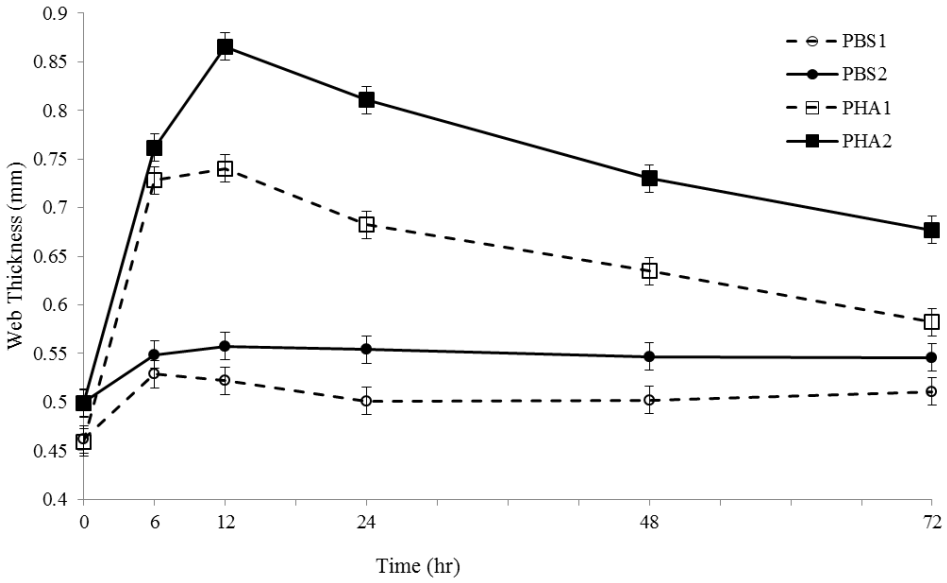

Figure 4.4 The effects of first and second injections of PHA (open and solid squares, respectively) and PBS (open and solid circles) on average (±SE) swelling response (mm) over time (hours).

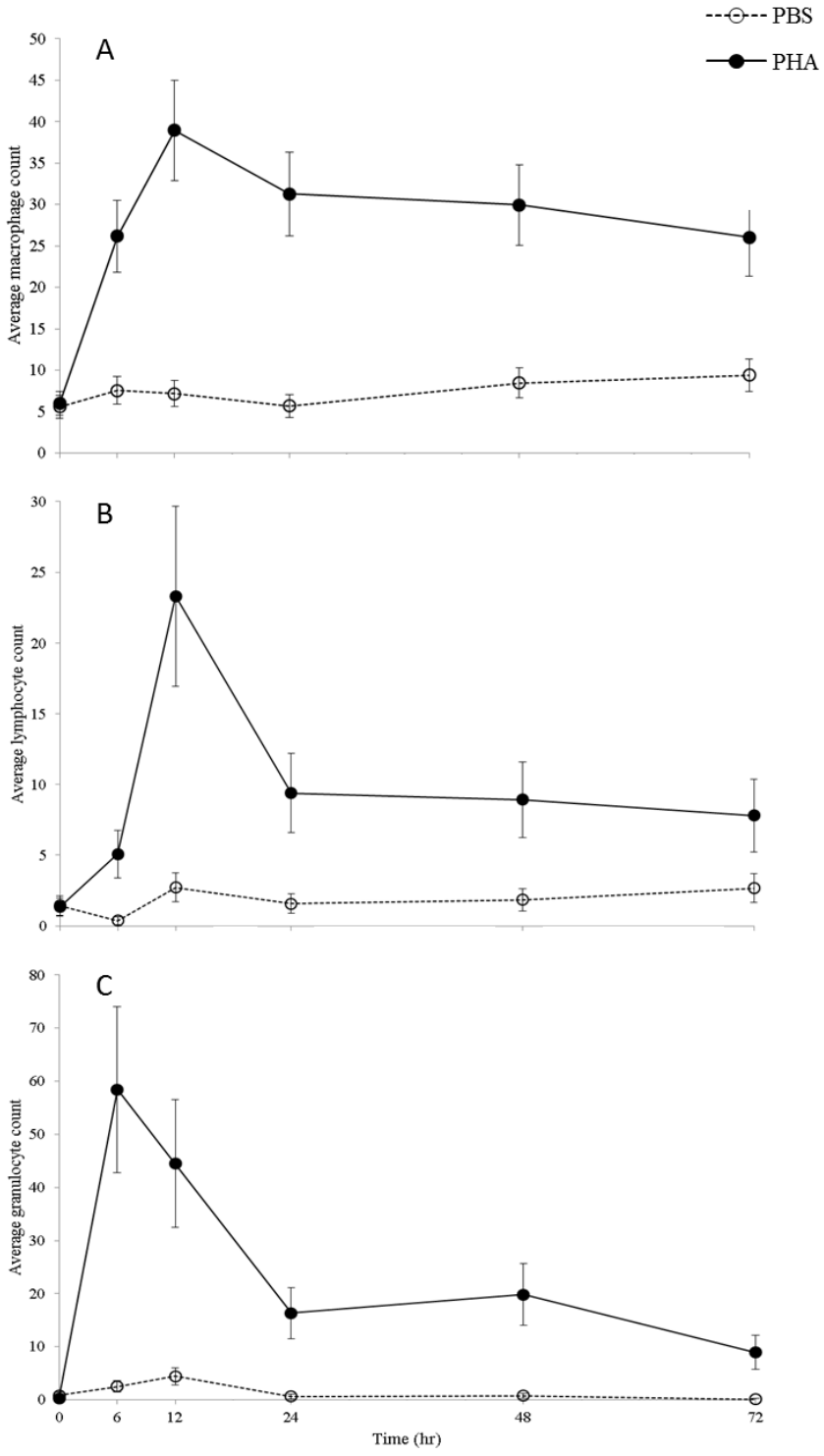
PBS
PHA
A
Average macrophage count
0
5
10
15
20
25
30
35
40
45
50
B
Average lymphocyte count
0
5
10
15
20
25
30
C
Average granulocyte count
0
10
20
30
40
50
60
70
80
0
6
12
24
48
72
Time (hr)

Figure 4.5 Changes in average (±SE) cell counts following administration with PHA (solid circles) and PBS (open circles). A. Macrophages, B. Lymphocytes, C. Granulocytes.

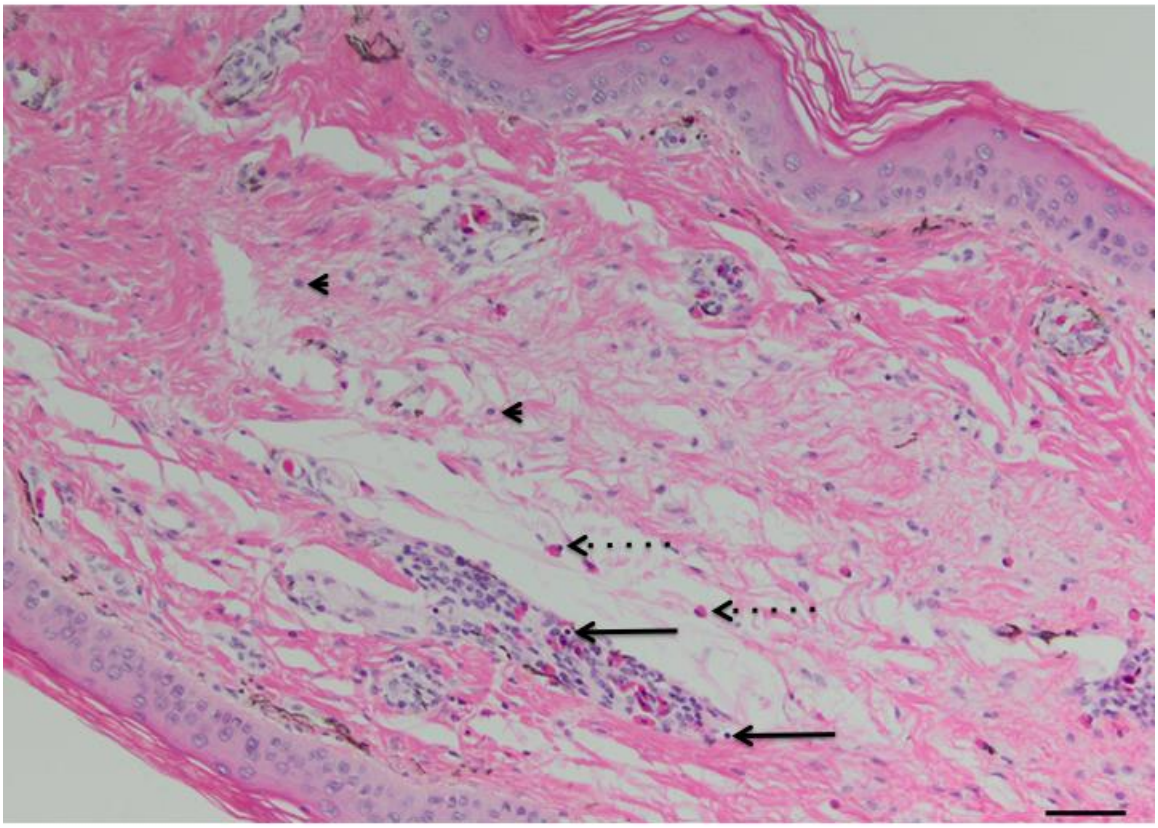

Figure 4.6 Photomicrograph (200×) of transverse section of PHA injected web at 12 hours post injection.

Scale bar = 50 µm. Examples of representative cell types are indicated by arrowheads for macrophages, dotted arrows for granulocytes, and solid arrows for lymphocytes.

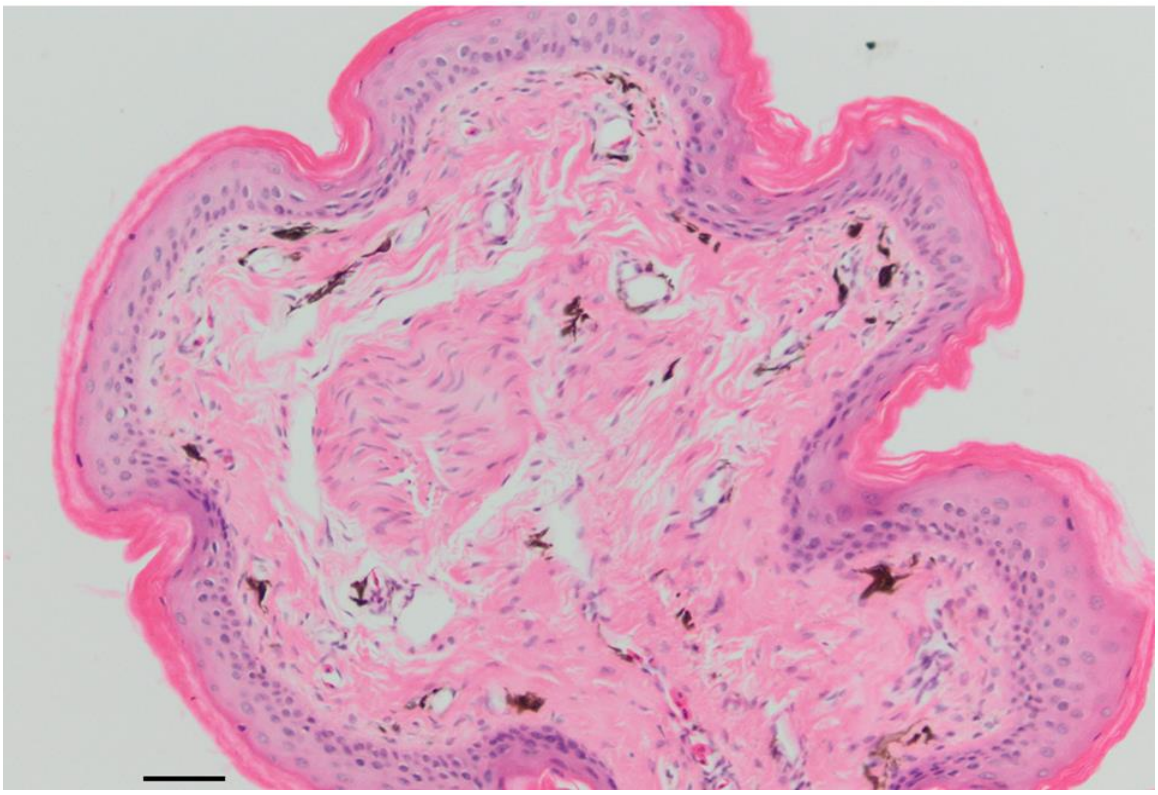

Figure 4.7 Photomicrograph (200×) of PBS injected web 12 hours post injection.

Scale bar = 50 µm.

CHAPTER 5

IN OVO AND *IN VITRO* SUSCEPTIBILITY OF AMERICAN ALLIGATORS TO AVIAN INFLUENZA VIRUS INFECTION [4]

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[4] Finger, J.W. Jr.*, B.L. Temple*, C.A. Jones, J.D. Gabbard, T. Jelesijevic, E.W. Uhl, R.J. Hogan, T.C. Glenn, and S.M. Tompkins. In press at the *Journal of Wildlife Diseases*. * These authors contributed equally to this manuscript.

Abstract

Avian influenza has emerged as one of the most ubiquitous viruses within our biosphere. Wild aquatic birds are believed to be the primary reservoir of all influenza viruses, however the spillover of H5N1 highly pathogenic avian influenza (HPAI) and the recent swine-origin pandemic H1N1 viruses have sparked increased interest in identifying and understanding which and how many species can be infected. Moreover, novel influenza virus sequences were recently isolated from New World bats. Crocodilians have a slow rate of molecular evolution and are the sister group to birds; thus they are a logical reptilian group to explore susceptibility to influenza virus infection and provide a link between birds and mammals. A primary American alligator (*Alligator mississippiensis*) cell line, and embryos, were infected with four low-pathogenic avian influenza (LPAI) strains to assess susceptibility to infection. Embryonated alligator eggs supported virus replication as evidenced by influenza virus M gene and infectious virus detected in allantoic fluid, and virus antigen staining in embryo tissues. Primary alligator cells were also inoculated with the LPAI viruses and showed susceptibility based upon antigen staining; however, the requirement for trypsin to support replication in cell culture limited replication. To assess influenza virus replication in culture, primary alligator cells were inoculated with H1N1 human influenza or H5N1 HPAI viruses that replicate independent of trypsin. Both viruses replicated efficiently in culture, even at the 30°C temperature preferred by the alligator cells. This research demonstrates the ability of wild-type influenza viruses to infect and replicate within two crocodilian substrates and suggests the need for further research to assess crocodilians as a species potentially susceptible to influenza virus infection.

Introduction

The emergence of zoonotic influenza A virus, including highly pathogenic avian influenza (HPAI) viruses H5N1 and pandemic H1N1 influenza viruses, has sparked worldwide interest in identifying and understanding which and how many species can be infected and serve as reservoir or vector species for influenza A virus. While influenza A virus has been well documented in mammalian and avian species, new viruses and host species are still being identified, most recently in bats (Tong et al. 2012; 2013). Recent research suggests that insects (Barbazan et al. 2008), reptiles (Davis and Spackman 2008; Huchzermeyer 2002), and amphibians are also susceptible to influenza infection (Barbazan et al. 2008; Davis and Spackman 2008; Huchzermeyer 2002; Mancini et al. 2004). Four species of crocodilian, the Chinese alligator (*Alligator sinensis*), smooth-fronted caiman (*Paleosuchus trigonatus*), broad-snouted caiman (*Caiman latirostris*), and Nile crocodile (*Crocodylus niloticus*), have evidence of influenza A susceptibility, with portions of the virus genome identified by PCR analysis of blood and serum of captive crocodilians (Davis and Spackman 2008). In addition, influenza C was identified by electron microscopy in the feces of farmed Nile crocodiles (Huchzermeyer 2003). To our knowledge, only samples from captive crocodilians have been analyzed; thus, data are deficient for wild crocodilians. Susceptibility to infection through direct inoculation of crocodilian tissues, cells, or live animals has not been investigated.

Avian species, mainly *Anseriformes* (e.g., ducks) and *Charadriiformes* (e.g., gulls), are the natural reservoirs of influenza A viruses (Hubalek 2004; Krauss et al. 2007). Evolutionarily, crocodilians are most closely related to birds (Crawford et al. 2012; Zardoya and Meyer 2001). Crocodilians and birds share several physiologic

features, such as egg structure and embryonic development (Deeming and Ferguson 1991), similar antibody isotypes (Warr et al. 1995), and homologous pancreatic polypeptides (Deeming and Ferguson 1991; Lance et al. 1984; Warr et al. 1995). Moreover, birds and crocodilians demonstrate similar susceptibility to some pathogens including West Nile virus, caiman pox virus, crocodile pox virus, adenoviruses, Newcastle disease virus, eastern equine encephalitis virus, and coronaviruses (Davis and Spackman 2008; Huchzermeyer 2003; Klenk et al. 2004; Ritchie 1995). Therefore, it is reasonable to hypothesize that crocodilians may be susceptible to other viruses endemic to birds, including influenza viruses, and could potentially serve as a reservoir or mixing vessel for these viruses.

Extant crocodilians are traditionally divided into three families including Alligatoridae, Crocodylidae, and Gavialidae, with the Alligatoridae including alligators and caimans (Janke et al. 2005; St John et al. 2012). American alligators live in proximity to and opportunistically feed on various avian species (Elsey et al. 2004). Thus, alligators may routinely be exposed to avian influenza virus (AIV) through the ingestion of infected tissues and inhalation of infectious particles during feeding (Reperant et al. 2008). Alligators are also exposed to bird excrement. As the fecal-oral route is considered the primary route of influenza virus transmission in aquatic birds (Wright et al. 1992), this may provide an additional route of infection for alligators.

The shared biological and ecological features of alligators and aquatic birds make alligators an important animal to investigate as a potential mixing vessel or reservoir for AIV. In addition, with increasing wild and farm populations along with habitat encroachment, human-alligator interactions are increasing. We used primary alligator

cells and alligator embryos to assess the susceptibly of American Alligators to influenza A virus infection.

Materials and Methods

Viruses

The low pathogenic avian influenza (LPAI) viruses used in this study were: A/chicken/Texas/167280-4/02 (H5N3), A/blue-winged teal/Louisiana/69B/87 (H4N8), A/mallard/Minnesota/Sg-00692/08 (H2N3), and A/blue-winged teal/Louisiana/Sg-00224/07 (H3N8). Notably, the H3N8 strain was isolated from the same region where the alligator eggs for this study were collected. The LPAI viruses were provided by David Stallknecht (University of Georgia, Athens, Georgia, USA). The human influenza A virus (A/WSN/1933 (H1N1)) is a mouse-adapted virus that replicated in cell culture without trypsin when serum is present (Goto et al. 2001). The HPAI virus, A/Viet Nam/1203/2004 (H5N1) was provided by Richard Webby (Saint Jude Children's Research Hospital, Memphis, Tennessee, USA). The viruses were propagated at 37°C in 9-11-day-old embryonated chicken eggs. Virus titers were assayed in Madin Darby Canine Kidney (MDCK) cells by 50% tissue culture infectious dose ($TCID_{50}$) assay (Mooney et al. 2013; Soboleski et al. 2011), and ranged from $10^{4.50}$ to $10^{6.24}$ $TCID_{50}$/mL. All experiments using live, HPAI viruses were approved by the institutional biosafety program at the University of Georgia and were conducted in biosafety level 3 enhanced containment following guidelines for use of Select Agents approved by the US Centers for Disease Control and Prevention.

Tissue Culture

Primary American alligator (*Alligator mississippiensis*) embryonic fibroblasts were established from a 41-51-day-old embryo. The embryo was chilled overnight at 4°C, dissected from the egg, washed with alpha-MEM (Gibco, Carlsbad, California, USA) and 1X antibiotics (100 IU/mL Penicillin G, 100 μg/mL Streptomycin, 0.25 μg/mL Amphotericin B), cut into 1x1 mm segments, and washed three times in MEM 1X antibiotic/antimycotic mixture. Segments were digested in a 1:10 dilution of Collagenase B (Roche, Indianapolis, Indiana, USA) in alpha-MEM cell culture media containing antibiotics (penicillin/streptomycin, Amphotericin B; alpha-MEM/AB media) for 3 hr. Suspensions were filtered through a 70 μm cell strainer (BD Falcon, San Jose, California, USA), washed with alpha-MEM/AB media, and centrifuged at 1500 x *g* for 15 min. Cells, which were presumed to be fibroblasts based on morphologic characteristics, were cultured in the alligator cell culture media (ACCM) for all experiments unless otherwise stated [ACCM: 175mM MEM (Gibco), Primocin (100μg/mL Invivogen, San Diego, California, USA), 2mM L-glutamine (HyClone, South Logan, Utah, USA), 1X Penicillin/Streptomycin Amphotericin B (CellGro, Manassas, Virginia, USA), 10% fetal bovine serum (FBS), 25mM HEPES (Gibco), and 1X sodium bicarbonate (7.5 μg/mL; Gibco), at pH 7.5 (Cuchens and Clem 1979)]. Cells were incubated at 30°C and 6% CO_2.

Egg Inoculation

Alligator eggs were collected from Rockefeller Wildlife Refuge (Cameron and Vermilion Parishes, Louisiana, USA, 29°73'N, 92°82'W). The University of Georgia Institutional Animal Care and Use Committee approved all protocols involving animals.

Eggs were incubated in a mixture of moist vermiculite and peat moss at 28-30°C and 90% humidity.

Viability was determined by candling. Viable eggs were segregated into four groups of 10 infected eggs and one group of 12 control eggs. Of the infected groups, five eggs were incubated at 33°C and another five at 36°C (33°C is the optimum temperature for normal alligator embryonic development; 36°C is the closest optimal temperature for virus replication without inducing embryonic lethality; Smith 1975). The control eggs were divided equally into temperature controls and vehicle controls (three untreated and three given vehicle and incubated at 33°C; three untreated and three given vehicle and incubated at 36°C). The injection site was disinfected with 70% ethanol prior to inoculation. Virus diluted (1:100) in phosphate-buffered saline (PBS) and antibiotics (100 IU Penicillin G, 100 μg/mL Streptomycin, 0.25 μg Amphotericin B/mL) were injected blindly (the allantoic fluid cavity was targeted, but not confirmed) using a sterile 24 mm 18 gauge needle. The injection site was sealed with Elmer's glue, and eggs were incubated for 5 days at indicated temperatures. Prior to extraction, eggs were chilled overnight at 4°C. Embryos were placed into 50 mL sterile conical tubes and filled with virus transport media [VTM; MEM, 100X Antibiotic/Antimycotic (10,000 I.U./mL Penicillin, 10,000 μg/mL Streptomycin, 25 μg/mL Amphotericin B), 50 μg/mL Gentamicin, 50 mg/mL Kanamycin, pH 7.4]. The allantoic fluid samples and whole embryos were stored at -80°C. The control embryos were cut to reveal viscera and stored at room temperature in 10% formalin.

Virus Titration

Virus titration of allantoic fluid and tissues from embryonated alligator eggs were assayed by $TCID_{50}$ assay using a cell-based enzyme linked immunosorbent assay (ELISA). Embryos were thawed at 4°C for 8 hr; tissues were extracted from embryos and homogenized in VTM using Qiagen Tissue Lyser (Germantown, MD, USA).

For the $TCID_{50}$ assay, MDCK cells plated in 96-well micro-titer plates were washed and replaced with MDCK infection medium [MEM, 2mM L-glutamine, 1X Antibiotic/Antimycotic (100 IU/mL Penicillin, 100 µg/mL Streptomycin, 25 µg/mL Amphotericin B), 50 µg/mL Gentamicin, 1µg/ml tosyl phenylalanyl chloromethyl ketone (TPCK) trypsin (Worthington, Lakewood, New Jersey, USA)]. Tissue homogenates were added in triplicate followed by 10-fold serial dilutions in the remaining rows. Negative (uninfected culture medium) and positive controls constituted the last row, with the positive controls infected with 500-750 $TCID_{50}$ of virus. Plates were covered and incubated for 36 or 72 hr (37°C, 5% CO_2) and fixed with methanol/acetone (80:20).

Plates were blocked overnight (5% nonfat dry milk, 0.5% bovine serum albumin (BSA), and 1X KPL wash buffer; KPL, Gaithersburg, Maryland, USA) at 4°C, washed three times, and incubated 1 hr with mouse anti-influenza nucleoprotein (NP) immunoglobulin G (IgG) (H16-L10; provided by Jon Yewdell, NIAID, Bethesda, Maryland, USA). After washing, anti-NP binding was detected using goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (1:10,000 dilution in KPL wash buffer), incubated in the dark at room temperature for 1 hr, followed by washing and detection using 1-Step Ultra TMB (3,3',5,5'-tetramentylbenzidine) ELISA substrate solution (Pierce, Rockford, Illinois, USA), following the manufacturer's directions. Sulfuric acid

was added to stop the TMB reaction and plates were read at 450 nm using a BioTek Powerwave plate reader (Bio-TEK, Winooski, Vermont, USA). For some samples, the virus titer was also estimated by measuring HA of MDCK culture supernatants with 0.5% turkey red blood cells (Mooney et al. 2013; Soboleski et al. 2011). Briefly, 0.05 mL of supernatant from each well of the $TCID_{50}$ plate was added to 0.05 mL of 0.5% red blood cells (diluted in PBS) and assayed for agglutination within 1 hr. The 50% endpoint was calculated via the Reed and Muench method (Reed 1938).

Immunofluorescence Staining

Alligator fibroblast cells were trypsinized using 0.05% Trypsin-EDTA and then plated at $7.5x10^5$ cells/mL in ACCM in 12-well plates. Once cells reached 80-90% confluence, the medium was removed and virus diluted (multiplicity of infection [MOI] + 0.05) in virus infection media (VIM; ACCM without FBS and HEPES plus 1μg/mL TPCK-trypsin, was added to each well. Inoculated plates were incubated for 3 hr at 30°C and 6% CO_2. Subsequently, virus infection media was removed, plates were washed with sterile PBS, and alligator cell growth medium (without trypsin) was added to each well. Plates were incubated at 33°C under 6% CO_2 for 24 and 72 hr, or at 37°C under 6% CO_2 for 24 hr. Following incubation, plates were washed in PBS and fixed for 20 min with methanol/acetone (80:20). Plates were blocked (5% FBS, 0.1% Tween20, and 1X KPL wash buffer) overnight at 4°C, washed three times, and incubated for 3 hr at room temperature with mouse anti-influenza NP IgG (H16-L10). Plates were washed and goat anti-mouse IgG FITC-conjugated in 1X PBS was added to each well. Plates were incubated at room temperature for 1 hr in the dark, washed, and 1μg/mL of 4',6-diamidino-2-phenylindole (DAPI) was added to each well to visualize cell nuclei. Plates

were incubated for 20 min at room temperature in the dark, washed, and examined using a Zeiss Axiovert 40 CFL fluorescent light microscope (Carl Zeiss Microscopy, Thornwood, New York, USA).

Assay For In Vitro Replication

Alligator fibroblasts were plated at 10^4 cells/well in ACCM in a 24-well plate. At 60% confluence, ACCM was removed and cells were washed three times with 1X MEM. Fibroblasts were infected with 100 plaque-forming units (PFU) of A/WSN/33 (H1N1) or A/Viet Nam/1203/04 (H5N1) diluted in ACCM without HEPES for 3 hr at 37°C, 5% CO_2. Following incubation, cells were washed three times with 1X MEM and 1 mL of ACCM without HEPES was added to each well and plates were incubated at 30°C, 6% CO_2. At 24, 48, and 72 hr, supernatants were collected, clarified by centrifugation, BSA added to 0.2%, aliquoted, and stored -80°C. Infectious virus titers in supernatants was determined by $TCID_{50}$ assay, as described, except that determination of influenza virus positive wells was determined by HA.

Immunohistochemistry

Formalin-fixed alligator organs (brain, trachea, lung, heart, liver, intestine, stomach, kidney, spleen, and pancreas) were dissected from embryos, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for histologic analysis. Other sections were rehydrated using standard procedures and stained using mouse anti-influenza A NP IgG biotin conjugated (Bioss Inc Woburn, Massachusetts, USA), followed by a streptavidin-HRP labeled secondary antibody. Lastly, 3,3'-Diaminobenzidine (DAB) was added to produce a brown precipitate in antigen positive tissues. Sections were reviewed and scored by a board certified pathologist (EWU).

Real Time Reverse Transcriptase (RT)-PCR

Total RNA was extracted from the liver, kidney, and a subset of allantoic fluid samples using a Qiagen RNeasy Mini Kit according to the manufacturer's protocol, except that a 250 µL aliquot of allantoic fluid was used in the extraction process (Spackman et al. 2002).

The cDNA synthesis and subsequent real-time RT-PCR were performed using a Qiagen One Step RT-PCR Kit on a Stratagene MX3000p or 3005p thermocycler (Life Technologies, Carlsbad, CA, USA) following the protocol of Spackman (2002). This is a well-established TaqMan® (Life Technologies, Carlsbad, CA, USA) protocol used as an influenza A virus diagnostic assay. The primer probe sequences are: M + 25, AGA TGA GTC TTC TAA CCG AGG TCG; M – 124, TGC AAA AAC ATC TTC AAG TCT CTG, and M + 64, FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA (Spackman et al., 2002). Each sample was run in triplicate, with influenza A/WSN/33 as a positive control.

Statistics

All statistical analyses were performed using Graph Pad Prism software (GraphPad Software, Inc., La Jolla, California, USA). A Mann-Whitney test or analysis of variance followed by Bonferroni's multiple comparison test were used to determine statistical significance. The level of significance for all data was set at $p<0.05$.

Results

In-Vitro Infection of Primary Embryonic Fibroblasts

Alligator primary embryonic fibroblasts were generated as described in the Methods and infected to determine susceptibility to AIV infection. Following inoculation

(MOI = 0.05) with A/blue-winged teal/Louisiana/Sg-00224/07 (H3N8), primary embryonic alligator fibroblasts were positive for virus nucleoprotein (NP; Figure 5.1). The NP antigen was present within the cytoplasm but was more strongly co-localized in the nucleus. Cells infected with AIV were positive for NP antigen after incubation at 33°C or 37°C at 24 hr postinfection. Infected alligator fibroblasts were also positive for NP at 72 hr postinfection at 33 C incubation. Infection with A/blue-winged teal/Louisiana/69B/87 (H4N8), A/chicken/Texas/167280-4/02 (H5N3), or A/mallard/Minnesota/Sg-00692/08 (H2N3) showed similar results (data not shown).

Infection of Embryonated Alligator Eggs with AIV

Embryonated alligator eggs were inoculated with one of the four LPAI (H5N3, H4N8, H3N8, or H2N3) strains to determine susceptibility to virus replication in ovo. Allantoic fluid and embryos were extracted 5 days postinoculation to assess the susceptibility to infection.

Infectious virus titers postinoculation exceeded input virus for all four strains indicated by virus titer levels in allantoic fluid of embryonated alligator eggs (Figure 5.2). The H5N3, H4N8, and H3N8 strains of influenza virus incubated at 33°C had mean virus titers significantly greater than input virus ($p<0.001$), with values of $10^{4.79}$ ($\pm10^{0.77}$), $10^{3.84}$ ($\pm10^{0.50}$), $10^{3.59}$ ($\pm10^{0.55}$) $TCID_{50}$/mL respectively (mean (±SEM)), whereas at 36°C these three strains had mean virus titers of $10^{4.84}$ ($\pm10^{0.65}$), $10^{4.35}$ ($\pm10^{0.15}$), $10^{4.39}$ ($\pm10^{0.57}$) $TCID_{50}$/mL, respectively. This suggests that temperatures chosen for replication had no significant impact on virus replication when cultured in alligator eggs. In contrast, the H2N3 virus demonstrated improved replication at the higher temperature, with the 33°C

group having a mean virus titer of $10^{3.49}$ ($\pm10^{0.67}$) $TCID_{50}$/mL compared to $10^{6.04}$ ($\pm10^{0.67}$) $TCID_{50}$/mL at 36°C (p=0.032). To validate these results, a subset of allantoic fluid samples were assayed in a standard $TCID_{50}$ assay, using HA as a measure of virus replication (Tompkins et al. 2007; Soboleski et al. 2011). The virus titer, as measured by HA endpoint, corresponded almost directly with the titers measured by cell-based ELISA (data not shown).

Virus genomic RNA was measured in a subset of allantoic fluid samples (n=5) from the 36°C alligator eggs. RNA was purified from allantoic fluid and M gene genomic RNA levels were measured using real-time RT-PCR. The mean cycle threshold (C_t) values from allantoic fluid samples (Figure 5.3) were below the negative threshold level of 34.79 (±0.17). H5N3-infected samples assayed had a mean C_t value of 13.97 (±0.15), while H3N8- and the H4N8- infected samples had mean C_t values of 15.38 (±0.13) and 15.25 (±0.06), respectively. The H2N3-infected samples had the lowest mean C_t value 13.23 (±0.27), corresponding to the highest titer measured in the $TCID_{50}$ assay (Figure 5.2). A/WSN/33 (H1N1), used as a positive control, had a mean C_t value of 15.67 (±0.37). Taken together, the infectious virus assay and M gene PCR data indicate influenza virus replication occurred within the alligator eggs.

AIV Infection in Embryonic Tissues

After confirmation of infection and virus replication of all four AIV strains in the allantoic fluid of embryonated alligator eggs, localization of replication was determined. Organs were extracted from embryos for H&E staining, IHC, virus culture, and RNA isolation.

All mock-infected embryos were negative for congestion and necrosis in all tissues and had no indications of antigen staining. However, one vehicle control at 36°C had fluid within lungs, resulting in nonspecific staining and so was excluded from further analysis (data not shown). In contrast, the alligator eggs infected with each of the four virus strains had NP staining predominantly in liver and kidneys and, in accordance with previous results, had higher levels of staining at 36°C. Embryos inoculated with each respective strain were positive for NP antigen staining in liver, kidney, or both. Likewise, positive staining was present at 33°C and 36°C. Embryos illustrated necrosis, congestion, and lesions in kidney and liver, and other tissues with all four virus strains (Table 5.1).

The IHC analysis indicated that the liver and kidney might serve as predominant sites for virus replication. However, attempts to measure tissue virus titers from embryos by $TCID_{50}$ were unsuccessful. To confirm the results obtained from the IHC, real time RT-PCR was utilized. Total RNA was extracted from both kidney and liver from the remaining embryos in each group and M gene RNA levels compared with controls. The mean control C_t values from the uninfected embryonic tissues were used to calculate the negative threshold value (36.40). The control values for the embryos incubated at 33°C were 40.00 (±1.03) for kidney (*n*=9) and 42.19 (± 1.14) for liver (*n*=9; mean (±SD)). Embryos incubated at 36°C had similar values of 40.65 (±1.03) for kidney (*n*=12) and 41.80 (± 0.75) for liver (*n*=12). The mean C_t value of liver and kidney samples from embryos infected with each of the four strains at two respective temperatures were below the negative threshold indicating the presence of M gene genomic RNA and virus replication in inoculated embryos (Figure 5.4). Temperature of incubation did not affect

virus replication for H4N8, H3N8, or H5N3 AIV. In contrast, consistent with previous results, H2N3-inoculated embryos incubated at 36°C had increased M gene RNA compared with embryos incubated at 33°C (Figure 5.4C).

Replication of Influenza Virus in Alligator Fibroblast Cells

As the LPAI viruses were able to replicate in alligator embryos, we next determined the ability of alligator primary embryonic fibroblasts to support the complete influenza virus lifecycle. Influenza virus generally requires addition of exogenous trypsin or another protease to activate the hemagglutinin of progeny virions, enabling multiple replication cycles (Shaw and Palese 2013; Wright et al. 2013). These proteases can be damaging to cells in culture, limiting the ability to assess influenza virus replication in many cell substrates. However, A/WSN/33 (H1N1) influenza can replicate efficiently without the addition of trypsin through sequestration of plasminogen in exogenously added serum (Goto et al. 2001). We infected alligator fibroblasts with 100 PFU of A/WSN/33 in media containing serum, cultured the infected cells at 30˚C and collected supernatants over time. The supernatants were assayed for infectious virus by $TCID_{50}$ assay. At 24 hrs post-inoculation there was limited, albeit measurable infectious virus detected (Figure 5.5A). The lower culture temperature was likely prolonging the eclipse phase of virus replication. However at 48 and 72 hr postinoculation, there was significantly more infectious virus (p=0.003), demonstrating replication of A/WSN/33 in alligator cells.

A hallmark of HPAI viruses is the ability to replicate in culture in the absence of trypsin. The hemagglutinin proteins of HPAI viruses have polybasic cleavage sites (in contrast to the monobasic cleavage sites found in most influenza viruses) that enable

cleavage and activation of the hemagglutinin protein by ubiquitous, furin-like proteases found in the Golgi complex of eukaryotic cells (Shaw and Palese 2013; Wright et al. 2013). To assess the ability of an avian influenza virus to replicate in alligators, we infected fibroblasts with 100 PFU of A/Viet Nam/1203/2004 (H5N1) and collected and assayed supernatants as before. Similar to the H1N1 virus, there was limited virus at 24 hr, but significant increases in infectious virus at 48 and 72 hr postinoculation ($p<0.001$), confirming that alligator cells are susceptible to AIV infection and can support the complete infectious lifecycle (Figure 5.5B).

Discussion

We demonstrated in ovo and in vitro susceptibility of American alligators to LPAI virus infection, with infection and replication occurring at temperatures much lower than wild-type AIVs would normally replicate (Massin et al. 2001; Massin et al. 2010; Scull et al. 2009). Measurement of virus load in allantoic fluid and culture supernatants indicated robust replication and production of virus progeny after infection with human influenza, LPAI, and HPAI viruses. While we demonstrated in ovo and in vitro susceptibility to AIV in alligators, the ability of AIV to replicate in live crocodilians remains unknown.

Primary alligator fibroblast cells were positive for NP after infection with each strain, demonstrating infection with multiple subtypes and strains. The primary alligator fibroblasts also supported production of infectious progeny viruses at 30°C and 37°C (data not shown). While the trypsin requirement that is common for growth of most influenza viruses in cell culture limited the viruses which could be assayed for replication, both human and avian influenza viruses replicated in the alligator cells, suggesting sialic acid moieties were present for hemagglutinin binding.

All four AIVs replicated within the embryonated alligator eggs, with all four strains infecting liver and kidneys. All virus-inoculated eggs produced high virus titers in the allantoic fluid. Virus titers were several logs greater than input titers for all four strains and at both temperatures, with the highest production at 36°C. Overall, these temperatures coincide within an optimal temperature range for alligator metabolic activity (Gans 1969). However, reptilian immune function is dependent upon temperature, with extreme temperatures perturbing function, thus, suppressed immune function may account for the increased virus titer at 36°C among certain strains (el Ridi et al. 1988). In contrast to increased titers at 36°C for the H2N3 virus, H5N3 virus replication was less affected by temperature, although the H5N3 virus also had the smallest increase in titer. However these data are not unexpected as concomitant in vitro and in vivo findings in a murine model illustrate that H5N3 replication may occur at temperatures as low as 33°C (Hatta et al. 2007).

Embryonic organs, notably liver and kidney, of infected eggs were positive for both genomic RNA and NP antigen, suggesting that infection and replication occurred within these organs. In contrast, the most common sites of infection in avian species are the lower respiratory tract and the gastrointestinal tract (Wright et al. 1992); therefore, we hypothesized that virus infection and replication would occur in similar tissues within embryonated alligators. However, as we restricted our study to in vitro and in ovo infections, the tissue tropism for infections in vivo remain unknown.

The viruses we used were all isolated from birds and geographic regions that overlap with alligator habitats in North America (Stallknecht et al. 1990). Thus it is possible for these viruses to intersect with alligator populations. The H5N3 LPAI virus

we tested was isolated from a poultry farm in Texas (Lee et al. 2004), and replicated in both primary cells and embryos at both low (33°C) and high (36°C) temperatures. A H5N1 HPAI virus replicated in alligator cells in culture and other HPAI viruses have been isolated from geographic regions overlapping with crocodilian habitats. While we did not assess the infection and transmission of influenza viruses in live crocodilians, the data suggest that alligators might be susceptible to influenza virus infection and that further study is warranted.

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Tables

Table 5.1. Histopathology from embryonic tissues.

Summary of histology and immunohistochemistry data from embryos extracted from inoculated alligator (Alligator mississippiensis) eggs. Numbers in parentheses are number positive/number tested. Dashes indicate no pathology or antigen staining found.

Virus	Head	Neck	Chest[a]	Abdomen[a]	NP Antigen[a]
Control[b]	-	-	-	-	-
H3N8 33°C	-	-	-	-	Lu, L ,K (1/2)
H3N8 36°C	-	-	Lu congestion (1/2) Lu necrosis (1/2)	-	L, K (2/2)
H4N8 33°C	-	-	Lu congestion (2/2)	K congestion (1/2)	Lu (1/2); L, K (2/2)
H4N8 36°C	Necrosis (2/2)	Necrosis (2/2) Bacteria (1/2)	Necrosis (2/2)	Necrosis (2/2)	Lu (1/2) L, K (2/2)
H5N3 33°C	-	-	-	-	L, K, I (1/2)
H5N3 36°C	Necrosis (1/2) Bacteria[c] (1/2)	Necrosis (1/2)	Necrosis (1/2)	Necrosis (1/2)	L, K (1/2)
H2N3 33°C	-	-	Lu congestion (1/2)	-	L (1/2)
H2N3 36°C	-	-	-	-	L, K (2/2)

[a] NP = nucleoprotein; L = Liver; Lu = Lung; K = Kidney; [b] Control embryos *n*=4. Infected embryos *n*=4 per virus strain. One control embryo in the vehicle control 36 C

group that inhaled fluid which resulted in non-specific staining (False positive). [c]

“Bacteria” indicates bacterial contamination.

Figures

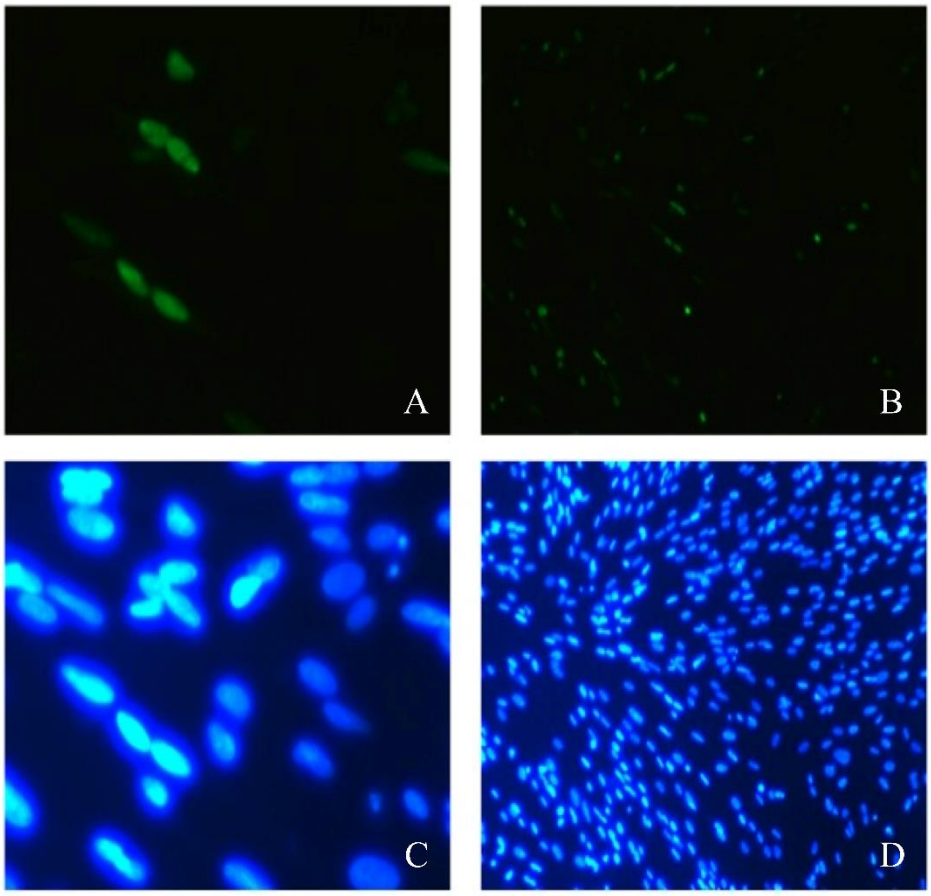

Figure 5.1: Immunofluorescent staining of nucleoprotein (NP) antigen in primary alligator (*Alligator mississippiensis)* fibroblasts infected with avian influenza virus, H3N8.

Positive staining of NP at an MOI of 0.05 24 hours post-infection at 33°C at 40X (A) and 10X (B) magnification. Co-localization of 4’,6-diamidino-2-pheylindole (DAPI) and NP 24 hours post infection at 33°C at 40X (C) and 10X (D) magnification.

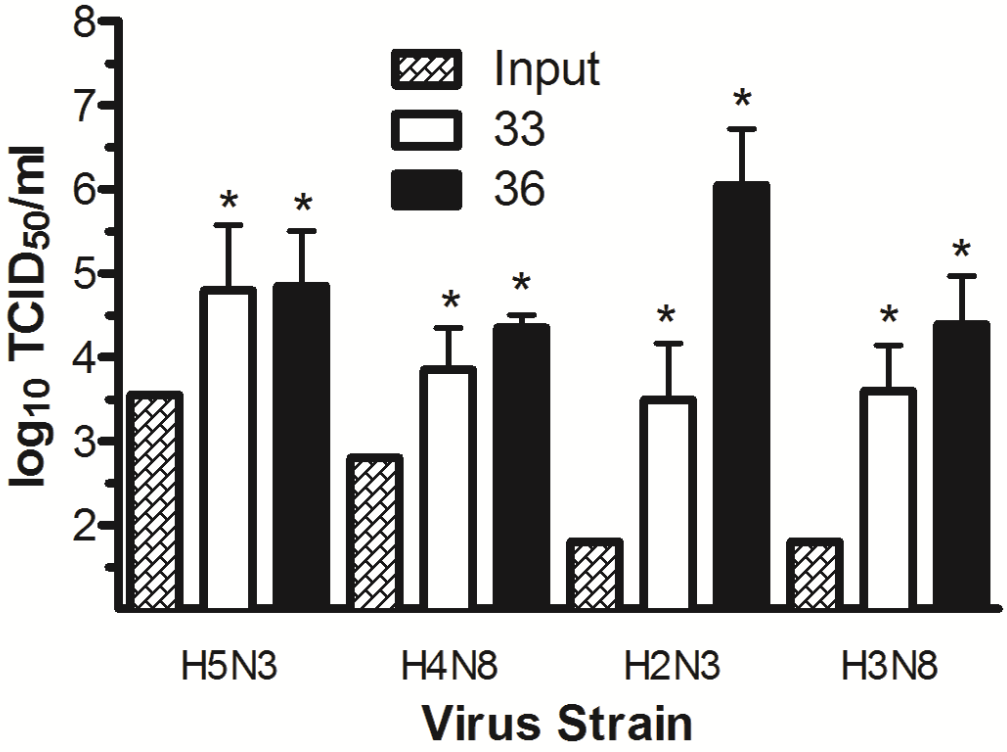

Figure 5.2: Mean (±SE) virus titer in allantoic fluid from embryonated alligator (*Alligator mississippiensis*) eggs.

Embryonated eggs (*n*=5/group) were inoculated with the indicated virus and incubated for 5 days at the indicated temperatures (33°C or 36°C). Allantoic fluid was collected and assayed for infectious virus by 50% tissue culture infectious dose ($TCID_{50}$) assay (*statistically significant, $p<0.05$).

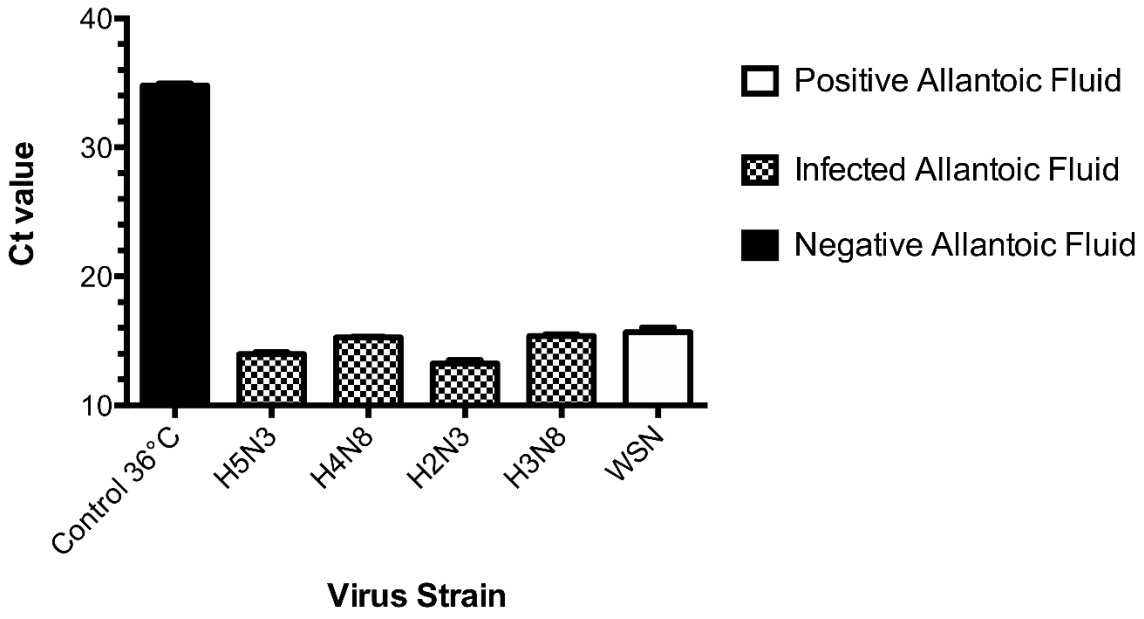

Figure 5.3: Real-time reverse transcription-PCR of M Gene levels from alligator (*Alligator mississippiensis*) allantoic fluid infected with low pathogenic avian influenza virus.

Embryonated eggs were inoculated with influenza virus and incubated for 5 days at 36°C. Allantoic fluid was collected and assayed for virus genome. Allantoic fluid was collected from naïve eggs as a negative control. Allantoic fluid from A/WSN/33-infected embryonated chicken eggs was used as a positive control. All infected samples were below the negative control cycle threshold (Ct) of 34.79. Control (*n*=9); H5N3, H4N8, and, H3N8 (*n*=6); H2N3 (*n*=3); and WSN (*n*=9).

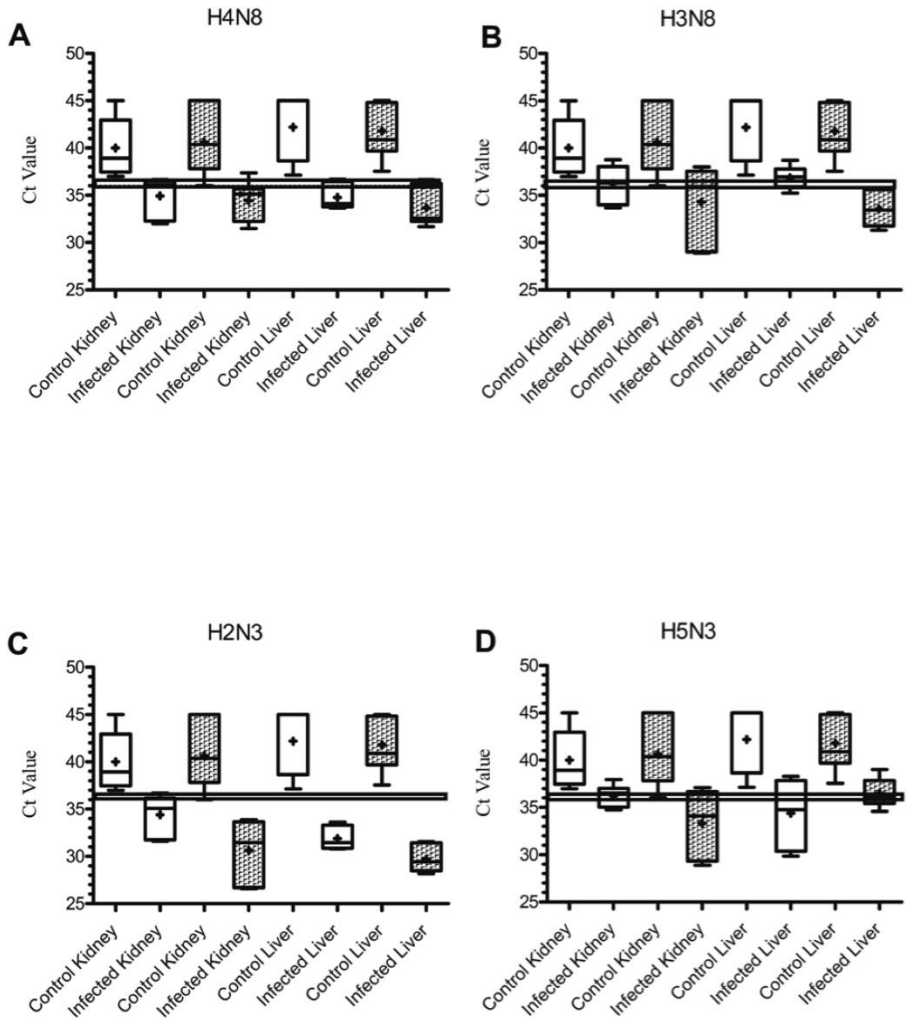

Figure 5.4: Reverse transcription-PCR of M gene in infected embryonic tissues.

Real-time RT-PCR results of Influenza A genomic RNA specific for M gene from kidney and liver samples of infected and uninfected alligator (*Alligator mississippiensis*) embryos. Embryonated eggs were inoculated with influenza virus and incubated for 5 days at 33°C or 36°C. Eggs were necropsied and tissues collected and processed for RNA as described in the materials and methods. Tissues were collected from naïve eggs as a negative control. All samples with cycle threshold (Ct) value >36.40 were classified as

negative for M gene. All samples with Ct <36.40 were classified as positive for influenza A genomic RNA. + indicates mean Ct value for all samples (n=9 except for 36°C control kidney and control liver where n=12). White box indicates an incubation temperature of 33°C; checkered box indicates incubation temperature of 36°C. A) H4N8 infected tissues. B) H3N8 infected tissues. C) H2N3 infected tissues. D) H5N3 infected tissues.

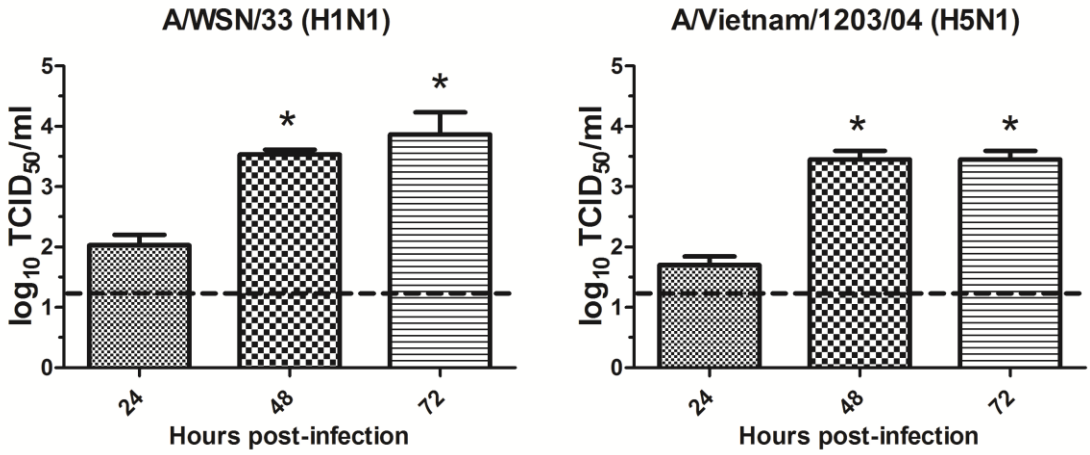

Figure 5.5: Infectious titers from inoculated primary alligator fibroblasts.

Primary alligator fibroblasts were inoculated with 100 PFU of (A) A/WSN/33 (H1N1) or (B) A/Vietnam/1203/04 (H5N1) influenza virus and incubated for 72 hrs at 30°C. Culture supernatants were collected at 24, 48 and 72 hrs post-inoculation and assayed for infectious virus by TCID50 assay as described in the materials and methods. Error bars indicate SEM of triplicate cultures (* $p<0.05$ compared to 24 hr time point).

CHAPTER 6

REFERENCE LEVELS FOR CORTICOSTERONE AND IMMUNE FUNCTION IN HATCHLING FARMED SALTWATER CROCODILES (*CROCODYLUS POROSUS*) USING CURRENT CODE OF PRACTICE GUIDELINES [5]

[5] Finger, J.W. Jr., P.C. Thomson, A.L. Adams, S. Benedict, C. Moran, and S.R. Isberg. Submitted to *General and Comparative Endocrinology*.

Abstract

Farming practices for crocodiles have changed over time in an attempt to reduce stress in the animals, which should improve their health and growth rates. To determine reference levels for on-farm stressors on immune responsiveness and growth rate, 253 hatchling crocodiles from 11 known-breeding pairs were repeatedly measured and blood sampled during their first year. Plasma corticosterone (CORT) was used to quantify the stress imposed by current farming practices and were found to be lower (mean 1.83 ± SE 0.16 ng/mL) than previously reported in saltwater crocodile hatchlings. Two tests of immune function were also employed. Innate constitutive immunity was assessed using bacterial killing assays (BKA) against two bacterial species: *Escherichia coli* and *Providencia rettgeri*, whereby the latter imposes considerable economic loss to industry from septicaemic mortalities. Although the bactericidal capabilities were different at Sampling 1 (approximately 4 months old; 32 ± 3% for *E. coli* and 16 ± 4% for *P. rettgeri*), the differences had disappeared by Sampling 3 (approximately 9 months old; 58 ± 2% and 68 ± 6%, respectively). Next, to assess immune responsiveness to a novel antigen, the swelling response caused by phytohaemagglutinin (PHA) injection was assessed but was only significantly different between Samplings 1 and 3 (5% LSD). There were no significant clutch effects for CORT or PHA but there were for both BKA traits. CORT was not significantly associated with growth (head length) or the immune parameters except for *P. rettgeri* BKA where higher CORT levels were associated with better bactericidal capability. As such, these results suggest that the crocodiles in this study are

not stressed, therefore endorsing the management strategies adopted within the Australian industry Code of Practice.

Introduction

It has been well-established that crocodile stress can be quantified using plasma corticosterone (CORT). As such, CORT has been used to assess various aspects of captive crocodilian propagation such as stocking densities (Elsey et al. 1990a, 1990b), water temperature (Turton et al. 1997), capture method (Franklin et al. 2003; Pfitzer et al. 2014), pen type (Isberg and Shilton 2013) and reproductive output (Elsey et al. 1990a, 1991; Lance and Elsey, 1986). Using this information, Codes of Practice for the captive propagation of crocodilians have been established (CFAZ 2012; LDWF and LSU 2011; NRMMC 2009; SABS Standards Division 2014). However, no study has attempted to quantify the baseline stress levels imposed under these conditions to validate the guidelines set out by the respective Codes of Practice.

CORT has most commonly been assessed against growth (Elsey et al. 1990a; Morici et al. 1997; Turton et al. 1997) which is an important economic indicator of production systems. Of further interest to producers is the well-defined negative relationship between CORT and immune function resulting in a retarded ability to ward off pathogenic infections under the imposed farming conditions. Morici et al. (1997) injected six month old alligators with slow release CORT pellets and observed a negative effect on growth and total leukocytes (lymphocytes, basophils, and eosinophils) with an increase in blood heterophil:lymphocyte ratio. Similarly in hatchling saltwater crocodiles, Turton et al. (1997) showed that experimental manipulation of water temperature invoked a CORT response and reduced overall leukocyte and lymphocyte counts. However, using

haematological parameters to assess herd health is not appropriate as there is no way to standardise an individual's exposure to antigens within the farming environment (Finger et al., 2013; Norris and Evans, 2000).

The production of farmed saltwater crocodile (*Crocodylus porosus*) skins is an important emerging industry in Australia (Isberg et al. 2004) demonstrating the success of sustainable-use conservation management strategies (Leach et al. 2009). However, because the industry is still in its infancy, baseline data to inform animal welfare standards are still being generated as an essential step towards ensuring economic viability and public acceptance of the industry. For example, juvenile mortalities are a continuing cause of economic loss in the industry, with hatchling crocodile mortality rates of 10-15% (Isberg et al. 2009). Some of these mortalities may be associated with farm-derived stressors inducing immunosuppression and disease susceptibility (Buenviaje et al. 1994; Camus and Hawke 2002; Isberg et al. 2009; Ladds et al. 1996; Morici et al. 1997; Turton et al. 1997), although no study has actually confirmed this premise.

This study was motivated by two main objectives. First and foremost, we set out to define baseline CORT levels imposed by *C. porosus* farming practices outlined in the current Code of Practice (NRMMC 2009) to both develop and refine crocodile welfare guidelines. Previous studies have already evaluated how capture and temperature manipulation may affect CORT in *C. porosus* (Franklin et al. 2003; Turton et al. 1997) so it was not our aim to experimentally manipulate such variables to determine the response to such stressors. Furthermore, to understand if individuals displayed consistently high or low CORT levels and if these levels changed over time, we sampled the same individuals

over three sampling periods which has not been previously attempted. Secondly, we aimed to better understand the relationship between CORT and immune function of farmed crocodiles using immune function measures that can be standardised in the context of the farming environment. To achieve this, we assessed the informativeness of two immune assays: bacterial killing assays (BKA) and phytohaemagglutinin (PHA) injection. The former is an *in vitro* assay to assess constitutive innate immunity in plasma (Demas et al. 2011; Matson et al. 2006; Merchant et al. 2003; Merchant et al. 2006; Millet et al. 2007), whereas the latter involves an *in vivo* injection of the antigenic lectin PHA, triggering a cellular immune response (local inflammation and swelling of the injection site) whereby subsequent measurements of the injection site can be used to determine immune responsiveness (Brown et al. 2011; Finger et al. 2013). We hypothesized that there would be a negative effect of CORT on the immune parameters investigated that would also be reflected in the growth of the individual.

Methods and Materials

Experimental Animals

Eggs obtained from 11 known captive breeding pairs were incubated under standard conditions (32 ± 0.5°C; 95-100% humidity) until hatching at Darwin Crocodile Farm, Noonamah, Northern Territory, Australia (Isberg et al. 2005; Miles et al. 2010). Upon hatching, the 253 crocodiles (mean 23.0 ± SE 3.65 hatchlings/clutch) were scute-cut for clutch and individual identification (Isberg et al., 2004) and subsequently housed in pens (Shed 1; 35 crocodiles in each pen; 116.5 cm in width and 209.5 cm in length) for three months prior to sampling to allow for acclimation. Water temperature was thermostatically controlled at 32˚C (± 1˚C), although air temperature was not regulated

within the enclosed shed. The rear half of each pen was covered with black shade cloth to provide security and heat retention, whilst the front half contained an open-air feeding platform. Crocodiles were fed to excess five times weekly between 13:30 and 15:30 hours with meat mince fortified with 2% vitamin/mineral premix (Monsoon Crocodile Premix, Winnellie, NT, Australia) and 1.5% calcium carbonate. Residual food was removed the following morning and pens were cleaned thoroughly with a chlorine-based detergent. Following the conclusion of the second sampling period (average age 189 days), the largest crocodiles were moved to larger pens (Shed 2; 55 crocodiles in each; 182 cm in width and 300 cm in length; water depth 14 cm) to reduce stocking density, although the basic pen setup and management regimes were the same. The remaining crocodiles in Shed 1 were dispersed between the empty pens to reduce their stocking densities. Throughout the study period, crocodiles were "graded" between pens to minimize the size variation and agonistic interactions (Morpurgo et al. 1993).

Sampling

At an average of 113, 189 and 275 days of age, respectively termed Sampling 1, 2 and 3 (Table 6.1), all 253 crocodiles were measured for head (HL) as described by Isberg et al. (2005) as an indicator of growth rate and sexed using blunt-nosed tweezers as described by Webb et al. (1984). Blood samples were taken from 40 animals for CORT measurement. These same samples plus blood samples from an additional 40 animals were used for the BKAs (total $n = 80$) as well as their toe-webs injected with PHA (total $n = 80$) as described below.

Corticosterone (CORT) Sampling

In an attempt to minimize handling and sampling stressors and to ensure baseline CORT levels were obtained, each sampling period was divided into four weeks. At Sampling 1, 10 animals were randomly selected each week (total 40) for repeated CORT assays and identified using metal-engraved small animal tags (National Band and Tag Co., USA). On sampling days, animals were left undisturbed for approximately one hour following pen cleaning to allow animals to re-acclimate. To standardize for possible circadian effects on CORT secretion (Lance and Lauren 1984), blood sampling always started at 10:00 hours when the animals were identified, caught and bled from the occipital sinus using a 23 gauge needle and 3 mL syringe. Samples from each individual were collected within three minutes from capture as recommended to obtain baseline levels (Romero and Reed 2005) as well as the cumulative time (CumTime; seconds) noted between entering the pen and obtaining the sample. Blood was stored in lithium-heparin tubes on ice for $\leq$ 20 minutes until centrifugation for one minute at 14,000 rpm. Plasma was used fresh for the bacterial killing assays described below with the remainder frozen (-20^{o}C) until the CORT assay was conducted.

CORT levels were determined using OCTEIA Corticosterone HS enzyme immunoassay kits (IDS Ltd., Tyne & Wear, UK) as per kit instructions at Berrimah Veterinary Laboratories (BVL, Berrimah, NT, Australia). To ensure samples were within kit detection limits (0.17-15 ng/mL), neat and 1:10 dilutions were run for each blood sample (Isberg and Shilton 2013). An appropriate model for conversion of percentage binding values to corticosterone in ng/mL was determined using the kit calibrator values.

CurveExpert (2009, version 1.4) curve fitting software was used to model the calibration curve with best-fit curves determined by their standard error and correlation coefficients.

Bacterial Killing Assay (BKA)

An aliquot of plasma was used to conduct bacterial killing assays (BKAs) within 20 minutes of blood collection. A modified protocol of Millet et al. (2007) was used to determine an individual's innate ability to neutralize *Escherichia coli* (ATCC 8739; Microbiologics USA) and *Providencia rettgeri* (ATCC 9250; Microbiologics USA). Fourteen microlitres of plasma was mixed with 266 μL of CO_2-independent media (Life Technologies Australia Pty Ltd, Australia) enriched with 4 mM L-glutamine (Life Technologies Australia Pty Ltd, Australia) at room temperature. Aliquots of each bacterial species were diluted with sterile phosphate buffered saline (PBS) to obtain working suspensions of ~20,000 CFUs/mL. Immediately following vortexing, 20 μL (~200 CFUs) of a working bacterial suspension was added to the blood/media solution, vortexed again, and 50 μL was immediately (time = 0 minutes) plated onto tryptic soy agar (TSA) plates (Edwards Group Pty. Ltd., Narellan, NSW, Australia). Thirty (*P. rettgeri*) and 60 minutes (*E. coli*) after initial bacterial addition, mixtures were re-vortexed and 50 μL of mixtures were added to new TSA plates. Control plates consisted of 280 μL L-glutamine enriched CO_2-independent media containing, 20 μL of working bacterial suspensions and no blood, and were also plated at 0 and 30 or 60 minutes depending on species. Plates were then inverted and were incubated at 35°C for 24 hours. Colonies were counted following 24-hour incubation. The killing ability for *E. coli*

(EcoliBKA) and *P. rettgeri* (ProvBKA) were expressed as the percentage of each bacteria killed using the formula (Millet et al. 2007) $\left(1 - \frac{\text{Colonies 30 or 60 minutes}}{\text{Colonies 0 minutes}}\right) \times 100$.

Phytohaemagglutinin (PHA) Administration

To assess an animal's ability to mount an immune response to a novel antigen, animals which were blood-sampled for CORT and BKAs also had the toe web between the first and second digit injected with 0.02 mL of 2 mg/mL PHA solution using a 0.3 mL syringe with a 29-guage needle as described by Finger et al. (2013). As a control, 0.02 mL of PBS was injected in the opposite foot. Prior to injection, web thickness was measured and was re-measured 24 hours post-injection using a pressure thickness gauge (Peacock G-1A; Ozaki Manufacturing Ltd., Japan). The 24-hour increase in web thickness (PHA-induced) was considered a representation of immune responsiveness with initial web thickness used as a covariate. Because PHA-induced swelling has been shown to increase with ensuing exposures (Finger et al. 2013), it was expected that subsequent injections would be greater due to immunological memory due to repeated exposure of an individual to the same antigen. To dissociate the effects of repeated injection (memory) from Sampling (seasonal), 32 animals were injected at Sampling 1 (Injection; Inj 1) and these same animals were reinjected at Sampling 2 (Inj2) and 3 (Inj 3). An additional 24 were injected at Sampling 2 (Inj 1) and again at Sampling 3 (Inj 2). A final 24 were only injected at Sampling 3 (Inj 1).

Disease Management

Standard management of Darwin Crocodile Farm is to submit any dead crocodiles to Berrimah Veterinary Laboratory (Berrimah, NT, Australia) for pathological

investigations, including antimicrobial susceptibility testing of pathogens causing disease outbreaks. Sick crocodiles are administered oral antibiotics when recommended by veterinarians, followed by two weeks of oral probiotics (Protexin; International Animal Health Products, NSW, Australia). The study animals were administered antibiotics twice during the study period. Throughout the first sampling period, all crocodiles (Shed 1) received sulfadimidine with trimethoprim (Sulprim; Ilium Veterinary Products Troy Laboratories, NSW, Australia) treatment for *Salmonella bovismorbificans* phage-type 13 which caused the deaths of two animals not related to this research (unpublished BVL necropsy report). Three and four weeks prior to the third sampling period, respectively, the crocodiles in Shed 1 and 2 finished a course of Sulprim to treat *Providencia rettgeri* outbreaks (unpublished BVL necropsy report). Both of these instances reported *Providencia rettgeri* as sensitive to sulphafurazole and trimethoprim/sulfamethoxazole but resistant to tetracycline.

Statistical Analysis

Both GenStat (Version 16.1) and ASReml (Version 3) were used. Univariate restricted maximum likelihood (REML) were used for initial modelling of the traits HL, CORT, PHA, EcoliBKA and ProvBKA. Both CORT and PHA were transformed to achieve normality and constant variance using natural logarithm (i.e. LnCORT and LnPHA). Fixed effects in the linear mixed model included either Age or Sampling (factor; j = 1, 2, 3) for all traits, the cumulative time to take blood sample (CumTime; in seconds) for LnCORT only, and initial web thickness (WT; mm) and Injection (Inj; k = 1, 2, 3) for LnPHA only. A time-adjusted CORT (CORTadj) predictor variable was created using the back-transformed standardised residuals from a linear regression of CORT

against time added to the mean CORT value for incorporation in the LnPHA, EcoliBKA and ProvBKA univariate analyses. Because the data are repeated measures, AnimalID was included as a random effect to link the observations of an individual. Clutch was also included as a random effect to account for any clutch effects (e.g. Turton et al. 1997) and a random spline model included for HL to account for nonlinearity of Age. The assumption of residual variance homogeneity between sampling periods were evaluated and accounted for where necessary. Observations were excluded if their residuals exceeded ±4 standard deviations from the mean. All interactions were included and a 5% significance level was chosen to evaluate the explanatory variable using backwards elimination. The significance of variance components were assessed using likelihood ratio tests again at the 5% level. All results are reported as back-transformed model-based means ± standard errors (SE).

Results

The terms found to be significant from each univariate models are shown in Table 6.2.

Corticosterone (CORT)

The overall mean CORT value from this study was 1.83 ± 0.16 ng/mL. The cumulative time (CumTime) between entering the pen, capturing the animal and procuring a blood sample significantly ($p < 0.001$) affected CORT (Figure 6.1). Sampling also had a significant effect on CORT ($p < 0.001$; Figure 6.2) and when compared to the first (mean ± SE; 1.33 ± 0.27 ng/mL) and third Samplings (1.36 ± 0.15 ng/mL),

individuals at the second Sampling period (~6 months old; 3.40 ± 0.30 ng/mL) had significantly increased CORT ($p < 0.001$; 5% LSD).

No significant Clutch effect was found. However, when the data were subset into the different Sampling periods, Clutch was significant ($p < 0.01$) for Sampling 1 but not for Samplings 2 or 3 ($p = 0.42$ and $p = 0.84$, respectively). AnimalID was non-significant between Samplings ($p = 0.55$; intraclass correlation = 0.05; Figure 6.3).

Bacterial Killing Assays (BKAs)

Bactericidal capacity of both *P. rettgeri* and *E. coli* increased with successive Sampling periods (Table 6.1). For *P. rettgeri*, the bactericidal capacity significantly increased by 36.2% at Sampling 2 (~6 months) and by 52.4% by Sampling 3 (~9 months) relative to Sampling 1 (~4 months; Figures 6.2 and 6.4), illustrating the profound effects of age on innate bacterial killing ability. Comparatively, the bactericidal capacity of *E. coli* increased by only 5% and 25.6% at Sampling 2 and 3, respectively. Interestingly, the initial killing ability of *E. coli* (32.2%) was double that of *P. rettgeri* (15.8%) although a similar end-point was achieved for both (57.9% and 68.1%, respectively; Figure 6.4).

CORTadj had a significant and positive effect (1.06 ± 0.04; $p = 0.006$) on *P. rettgeri* bactericidal ability but no effect on *E. coli* ($p = 0.631$). As per the deviance difference test, Clutch had a significant effect on killing of both *E. coli* and of *P. rettgeri*.

PHA

PHA-induced swelling was significantly affected by initial web thickness (2.43 ± 0.39 mm/mm; $p < 0.001$) and Sampling period ($p = 0.038$; Figure 6.2 and 6.4). Swelling increased over the consecutive Sampling periods with averages of 0.80 ± 0.02 mm, 0.83

± 0.02 mm and 0.89 ± 0.02 mm, respectively (Figure 6.4), although only Sampling 3 was significantly elevated relative to Sampling 1 (5% LSD). AnimalID was not significant (p = 0.34) and neither was Clutch (p = 0.10). Unexpectedly, repeat injections (Inj) were not significantly different to the primary response.

HL

HL significantly increased by 0.15 ± 0.01 mm HL/day ($p < 0.001$). Clutch was also significant but there was no effect of CORTadj (p = 0.98) on HL. However, when the same univariate model was used to investigate each Sampling period individually, CORTadj significantly and negatively affected hatchling growth in Sampling 1 only (p = 0.04; Sampling 2 p = 0.99; Sampling 3 p = 0.40).

Discussion

Corticosterone

The overall mean CORT value from this study was within the range reported for captive juvenile *A. mississippiensis* (Elsey et al. 1990a; Lance and Lauren 1984) but significantly lower than previously described for captive hatchling *C. porosus* (Shilton et al. 2014; Turton et al. 1997). However, all previous hatchling *C. porosus* studies were conducted on animals less than seven months of age when CORT appears to be more variable (Table 6.1; Figure 6.2 and Figure 6.3). This is the first study to repeatedly measure CORT over an extended time period to generate individual profiles (Figure 6.3). More so, this is the first study to quantify the level of stress imposed by commercial crocodile production guidelines.

The cumulative time (CumTime) between entering the pen, capturing the animal and procuring a blood sample significantly affected CORT (Figure 6.1). Previous studies

have also demonstrated the significance of this effect (e.g. Isberg and Shilton 2013; Romero and Reed 2005) and future studies should take this into account when sampling group-housed animals to obtain reliable baseline CORT plasma levels as not all studies have attempted to account for this effect (Shilton et al. 2014; Turton et al. 1997).

We hypothesized that individuals would display either consistently high or low CORT values given their (non-)adaptation status to farm husbandry (Shilton et al. 2014). This was not observed but instead stochastic profiles resulted (Figure 6.3). Previous research has suggested that CORT levels in crocodilians may be related to body condition (Shilton et al., 2014), stocking density (Elsey et al., 1990a), capture (Franklin et al. 2003; Lance and Elsey 1986), temperature (Turton et al. 1997), and/or time of sampling (Lance and Lauren 1984). However, these potential confounders were minimized by providing excess food, keeping animal sizes similar within pens and within the recommended stocking density guidelines (NRMMC 2009), providing heated water, obtaining blood samples within three minutes of capture (Romero and Reed 2005) and negating circadian effects by consistently commencing sampling at 10:00 hours. Furthermore, subjects were exposed to identical daily regimes (i.e. feeding, cleaning) from time of hatch (habituation), even on sampling days. Despite minimizing and standardizing for these influences, the observed stochastic individual CORT values show that not all stressors are simply accountable. Irrespective, the CORT values reported herein define the stress imposed by current management practices to allow producers a point of reference when manipulating their management practices.

Means were unequal between Samplings. Sampling 2 displayed the highest CORT response and there are a few possible reasons for this difference. First, previous

studies have illustrated that CORT can be influenced by environment (Romero 2002; Turton et al. 1997; Tyrrell and Cree 1998) and Sampling 2 (September/October) coincided with the onset of "the build-up", a time of high temperatures and humidity but little to no rain (Webb et al. 1991). Despite the water temperature being thermostatically-controlled (32^{o}C), the rise in air temperature and humidity may have increased metabolic rates inducing greater competition for food and space (Morpurgo et al. 1993). Second, the biomass of animals within each pen was highest at Sampling 2 and animal numbers were subsequently reduced thereafter. Elsey et al. (1990a) reported higher CORT with higher stocking densities in juvenile American alligators and while the number of animals within the pens had not changed, the animals had grown significantly so again the competition for resources had increased (Morpurgo et al. 1993).

In addition to unequal means, heterogeneous variances were also observed whereby both Sampling 1 and 2 had double the variance of Sampling 3. The unequal variance can be interpreted that crocodiles within the same environment respond differently to similar stressors and again, there could be a few reasons for this. First could be the maternal transfer of CORT *in ovo*. Avian studies have shown that increased glucocorticoid exposure *in ovo* may affect a multitude of factors including reproductive hormones (Henriksen et al. 2011), growth (Hayward and Wingfield 2004) and offspring survival (Saino et al. 2005). Whilst reduced nesting success has been associated with increased CORT in adult female alligators (Elsey et al. 1990b), the maternal transfer of CORT *in ovo* has yet to be demonstrated in crocodilians (see Shilton et al. 2014). However, if CORT is transferred in the yolk similar to birds (Almasi et al. 2012; Saino et al. 2005), the age when CORT is measured post-hatch may explain the unequal residual

sampling variation observed during this study period. If the latter is true though, a significant clutch (maternal genetic) effect would have been expected but consistent with Turton et al. (1997) this was not observed. However, when we subset the data, a significant Clutch effect was present for Sampling 1 adding support to this hypothesis. Alternatively and/or confoundingly, the difference in CORT variation could represent an individual's ability to adjust to changing seasonal or on-farm conditions as previously described. Further work needs to be conducted to dissociate these factors but highlights the need to make these observations at more than one time-point in an individual's lifetime.

The similarity between reported *A. mississippiensis* CORT concentrations and these results for farmed *C. porosus* suggest satisfactory on-farm conditions and welfare standards. Whilst it could also be argued that our results may reflect desensitization to repeated, chronic stressors, it has previously been shown that runts from the same farming environment have very high CORT levels relative to those growing normally (Shilton et al. 2014). As such, desensitization can somewhat be dispelled.

BKA

Deaths caused by bacterial septicaemias cause significant economic losses for the Australian crocodile industry (Isberg et al. 2009). Usually, these deaths follow manifestation of a reputed stressor such as capture or a failure in husbandry protocols (Camus and Hawke 2002; Huchzermeyer 2003; Isberg et al. 2009; Ladds et al. 1996). Septicaemias caused by *P. rettgeri* (Buenviaje et al. 1994; Camus and Hawke 2002; Ladds et al. 1996) remain the most prevalent (Isberg et al. 2009) while *E. coli* was selected for comparison with previous studies rather than as an economically important

cause of death although cases have been reported in *C. porosus* (Isberg et al. 2009; Sultana et al. 2012).

Our data indicate young crocodiles have a more established innate ability to neutralize *E. coli* compared to *P. rettgeri*, however by nine months of age, this ability has been equilibrated/equalized. The reason for this remains unknown, however these results are consistent with observations on the farms where younger animals succumb to *P. rettgeri* septicaemias more regularly than older (>1 year old) animals (Isberg et al. 2009) and are rarely diagnosed with *E. coli* septicaemias (unpublished BVL necropsy reports).

As the immune system of reptiles is intimately tied to the environment and climate (Merchant et al. 2003; 2006; Zimmerman et al. 2010a; 2010b), the aforementioned observations of a Sampling effect may have been due to seasonal fluctuations in temperature, photoperiod, humidity or precipitation (Webb et al. 1991). For example, *in vitro* crocodilian innate bactericidal capabilities are reliant upon temperature (reviewed in Finger and Isberg 2012) with higher temperatures increasing bactericidal efficacy up to a set point (e.g. Merchant et al. 2003). Furthermore, environmentally-induced alterations (i.e. climate) in immune capabilities have been shown in other reptiles (Zimmerman et al. 2010b). As such, the increased ambient environmental temperatures experienced by the animals at Sampling periods 2 and 3 may have accounted for increased bacterial killing efficiency. On the other hand, the sequentially increased lytic capacity may be due to enhanced immune function associated with early ontogeny observed in some reptiles (see El Deeb and Saad 1990). Similar to birds (Garnier et al. 2012; Liu and Higgins 1990), reptilian maternal transfer of yolk derived-antibodies may confer immunocompetence for the first few months of life or

longer (Schumacher et al. 1999). Thereafter, individual-developed immunocompetence is relied upon.

The positive effect of CORT on *P. rettgeri* BKA refutes our hypothesis, although this is not unprecedented and has been shown in birds (Buehler et al. 2008; Merrill et al. 2014) and amphibians (Hopkins and Durant 2011). Although stress is often viewed in a negative light, a certain level of glucocorticoids is necessary for basic physiological functioning (Rooney and Guillette 2001). As such, since the CORT levels herein are relatively low, the higher CORT levels may have been acutely immunostimulatory (reviewed within Dhabhar 2002). Since *P. rettgeri* is ubiquitous on crocodile farms (Isberg et al. 2009; Ladds et al. 1996), a positive association with CORT would provide a survival advantage. Interestingly, there was no significant relationship between CORT and EcoliBKA similar to the findings of Rubenstein et al. (2008) in superb starlings (*Lamprotornis superbus*).

E. coli was used for comparative purposes with other studies. In chickens, cockatiels, and American kestrels, incubation with whole blood resulted in 68, 81 and 95% bacteria killed, respectively (Millet et al. 2007) although no ages were given. In non-stressed adult marine iguanas (*Amblyrhynchus cristatus*), bacterial killing ability was greater than 70% in the non-breeding season (French et al., 2010). *E. coli* has also been used to investigate the antimicrobial action of crocodilian serum (Merchant et al. 2003; 2006) although a different method was used (spectrophotometry). Following incubation of *A. mississippiensis* serum with a comparable *E. coli* concentration used herein (10^4 CFUs/mL), Merchant et al. (2003) observed a 97% decrease in *E. coli* survival but no details of sex, age or time of sampling were provided. Merchant et al. (2006) continued

by assessing the serum bactericidal ability of all 23 crocodilian species against a range of bacteria using the "zone of inhibition" method and, in particular, found a range in capability for *E. coli* ranging from 0 mm (*Crocodylus siamensis* and *Osteolaemus tetraspis*) to > 6 mm (eight species including *Crocodylus porosus*). However, using the "zone of inhibition" method provides no quantification of bactericidal effects because it is difficult to differentiate between bacteriostatic and bactericidal effects, and again no details of sex, age or time of sampling were provided for comparison. As such, the lack of replicates, relatedness of animals, age structure and details of sampling (e.g. season, temperature, sex, nutritional status) prevent direct comparison with these results and the repeated sampling methodology employed herein shows that the innate bactericidal ability of crocodile hatchlings is not static.

The significant clutch effect on bacterial killing ability suggests that quantitative differences in maternal-derived antibodies or differences in underlying innate immune genes may influence immunity (Bhide et al. 2009; Staszewski and Siitari 2010). Although yet to be investigated in crocodilians, maternal transfer of antibodies provides immune defence for the first few months of life in birds and other reptiles (Garnier et al. 2012; Muller at al. 2004; Schumacher et al. 1999). For example, *Mycoplasma agassizii*-infected female desert tortoises (*Gopherus agassizii*) transferred significantly higher *Mycoplasma*-specific antibody titres to their offspring, which were still detectable after one year compared to seronegative females (Schumacher et al. 1999). On the other hand, constitutive innate defences, such as those assayed in BKAs, are germ-line encoded (i.e. complement proteins, pattern recognition receptors). Therefore, polymorphisms or

mutations, such as those in complement genes or pattern recognition receptors, may influence bacterial lysis (see Bhide et al. 2009).

PHA

PHA was used in this study to evaluate the immune responsiveness (i.e. swelling) following injection with a novel antigen (Finger et al. 2013). In contrast to the BKAs, PHA injection may measure both subsets of the immune system. The primary PHA injection mainly measures the pro-inflammatory, phagocytic potential of the innate immune system, whilst the swelling from subsequent injections may provide insight into acquired immunity via immunologic memory (Finger et al. 2013; Martin et al. 2006; Salaberria et al. 2013; Vinkler et al. 2010). Unexpectedly and contrary to our previous findings (Finger et al. 2013), the animals used herein showed no immunologic memory (i.e. subsequent injections did not increase swelling) although there was a significant effect of Sampling implying an age and/or seasonal response to antigenic injection. The lack of an injection effect might reflect the longer interval between injections (12 weeks) compared to three weeks used by Finger et al. (2013). More so, injection number may be confounded within Sampling (Zimmerman et al. 2013a; 2013b). In fact, whilst PHA has been used as an indicator of immunity in a multitude of studies, it is important to emphasize that cautious interpretation is required due to a host of exogenous and endogenous factors that may affect its response including nutritional status, stress, reproduction and/or season (see Berger et al. 2005; Finger et al. 2013; Kennedy and Nager 2006; Martin et al. 2006; Sorci et al. 1997; Zimmerman et al. 2010b). Furthermore, the functional aspects of the test itself present hazards to interpretation. Mechanistically,

PHA-induced swelling is not restricted to T-lymphocytes and a plethora of leucocytes infiltrate the tissue as well (Brown et al. 2011; Finger et al. 2013; Martin et al. 2006).

No significant effects of CORTadj were observed on PHA swelling, further suggesting the crocodiles were not stressed. Similarly, CORT had no effect on PHA-induced swelling in tropical house sparrows either (*Passer domesticus*; Martin et al. 2005). Alternatively, our results may reflect a short-term responsiveness to PHA-induced swelling that may not incorporate the negative effects of chronic stress on immunity. For example, Roberts et al. (2007) showed that high CORT induced by 20 minutes of restraint had no effect on PHA in zebra finches (*Taeniopygia guttata*).

Growth Rate

Head length (HL) was used as the indicator of crocodile growth and was significantly influenced by age and clutch of origin (Isberg et al. 2005; Turton et al. 1997). The null effect of CORT on HL in this study is different from previous studies showing higher CORT levels to have a detrimental impact on growth (Elsey et al. 1990a; Turton et al. 1997). However, the CORT levels presented here (Table 6.1) are lower than previously reported in saltwater crocodiles (Isberg and Shilton 2013; Shilton et al. 2014; Turton et al. 1997), thus again suggesting the level of husbandry stressors to be negligible.

When the data were subset into separate Sampling periods, a significant effect of CORT was found. Similarly, nestling zebra finches treated with CORT exhibited suppressed body size 30 days after hatching but no effect was observed at 60 or 90 days post hatch (Crino et al. 2014). Whilst increased CORT post-hatch may reduce growth, this cost may be offset by other benefits such as increased foraging, aggression, food

intake, or increased risk taking behaviour both in early and adult life (Kitaysky et al. 2003; Zimmer et al. 2013). Furthermore, increased CORT in hatchlings may reduce predation risks (see Hayward and Wingfield 2004). Our results add credence to the anecdotal belief that the first three months post hatch may be the most critical as hatchlings adjust to their environment and establish hierarchies (Brien et al. 2013). Furthermore, these results may add additional strength to the hypothesis of maternal CORT transfer *in ovo*.

Movement of Animals Following Sampling 2

Grading is an essential component of crocodile farming to reduce the size variation within a pen and reduce competition for resources to facilitate growth (Brien et al., 2013; Morpurgo et al. 1993). Because this study was designed to understand the baseline CORT and immune values using current husbandry practices, we necessarily graded animals when necessary based on size and not by randomization.

As it has previously been shown that high-density housing increased stress and subsequently reduced growth in crocodilians (e.g. Elsey et al. 1990a) and our specific aim was to determine how regular practices and regimes on a crocodile farm may contribute to baseline levels of plasma CORT, we removed larger animals from Shed 1 after Sampling 2 to decrease stocking densities. We were unable to assess the effect of Shed on traits, because the movement of individuals was not random. We biased the movement to mainly include larger individuals. Thus, Shed (during Sampling 3) was confounded within HL and could not be used as a factor in analysis.

Antibiotics and Disease Management

Throughout certain portions of our study (see Methods), crocodiles were administered antibiotics following post-mortem pathological confirmation of bacterial-induced mortality. Whilst such administration was necessary to control and prevent mortality, antibiotic exposure may have affected the results presented above as the use of antibiotics at prior samplings may have enhanced bacterial killing ability at later samplings. However, in contrast to reported observations of decreased innate immunity following bacteriostatic antibiotic exposure (Brandl et al. 2008; Kristian et al. 2007), we observed sequential increases in bactericidal ability at each sampling period. Similarly, whilst antibiotic exposure has been shown to suppress PHA-induced leucocyte mitogenesis *in vitro* (Grondel et al. 1986; Nasjleti and Spencer 1968), we saw an increase at each Sampling. Furthermore, it is important to emphasize that the functional and interpretable effects of *in vivo* vs. *in vitro* PHA exposure are markedly disparate (see Kennedy and Nager 2006; Martin et al. 2006; Vinkler et al. 2010). Nevertheless, disclosure of antibiotic administration is warranted as it may have influenced the observed results.

Conclusions and Future Studies

This is the first study to repeatedly measure and examine the interactions between CORT, immune parameters (BKAs and PHA injections) and growth in farmed *C. porosus* hatchlings or any other crocodilian species. The results suggest that the crocodiles were not inherently stressed and, therefore, endorse the management strategies adopted within the Australian industry Code of Practice (NRMMC 2009). Only ProvBKA was influenced by CORT but with a positive association. Otherwise, growth

(HL), innate immunity against *E. coli* (EcoliBKA) and cellular immunity (PHA) were not affected by CORT (CORTadj). Likewise, Turton et al. (1997) found no relationship between CORT and two other immunological indices (antibody titre and total leucocytes) in hatchling *C. porosus*. On the other hand, the immunological tests employed to date may not provide the best insight into stress-immune interactions in *C. porosus* remembering that these interactions are not always straightforward and other factors may affect the outcome, such as duration of stressor exposure or physiological status (i.e. nutritional or reproductive status) (Berger et al. 2005; French et al. 2010; Matson et al. 2006; Tieleman et al. 2005). Further research is necessary to determine which immunological techniques are the most suitable for assessing standardised crocodilian immunity and evaluating stressor-immune interactions.

The significant effect of Sampling on all traits (CORT, ProvBKA, EcoliBKA, and PHA) shows the non-static nature of these traits. Further investigations should be conducted to disentangle the effect of age from other environmental (e.g. air versus water temperature, humidity) and maternal (e.g. *in ovo* transfer of glucocorticoids) effects using experimental manipulations.

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Tables

Table 6.1 Model-based means ± standard error of head length (HL), time-adjusted corticosterone (exponentiated CORTadj; ng/ml), integer bacterial killing ability of *E. coli* (EcoliBKA) and *P. rettgeri* (ProvBKA), and web thickness increase to PHA injection (exponentiated PHA, mm) over three sampling periods in hatchling saltwater crocodiles.

S	M	Season[a]	Age (days)	HL (mm)	CORTadj (ng/ml)	EcoliBKA	ProvBKA	PHA (mm)
1	June/July	Cool, dry	112.9 ± 0.6	67.83 ± 0.63	1.33 ± 0.27	0.32 ± 0.03	0.16 ± 0.04	0.80 ± 0.02
2	Sept/ Oct	Hot, dry	188.9 ± 0.8	76.36 ± 0.81	3.40 ± 0.30	0.37 ± 0.03	0.52 ± 0.05	0.83 ± 0.02
3	Nov/ Dec	Hot, wet	275.1 ± 1.2	89.61 ± 1.09	1.36 ± 0.15	0.58 ± 0.02	0.68 ± 0.06	0.89 ± 0.02

(S=Sampling; M=Months). [a] Webb et al. 1991

Table 6.2 Model-based means (±SE) and significance of terms from the univariate restricted maximum likelihood (REML) modelling on the back-transformed scale. Levels of significance are shown as $p < 0.05$(*), $p < 0.01$ (**) and $p < 0.001$ (***). A dash (—) indicates that the term was not significant ($p > 0.05$), whilst N/A specifies that terms were not applicable to the trait. Model-based means have been back-transformed for CORT and PHA. Responses with heterogeneous sampling variances are indicated by a ≡.

Traits	Fixed effects							Variance componer	
	mu	Age (days)	Sampling	Time (secs)	CORTAdj (ng/mL)	Initial Web Thickness (WT)	Pen	spline	Clutch
HL	48.19 ± 2.14	0.15 ± 0.01***	N/A	N/A	—	N/A		67.80 ± 42.11*	2.30 ± 1.16*
CORT	1.83 ± 0.16	—	***	1.00 ± 0.00***	N/A	N/A	—	N/A	—
ProvBKA	0.45 ± 0.04	—	***	N/A	0.06 ± 0.02**	N/A	***	N/A	0.01 ± 0.01*
EcoliBKA	0.42 ± 0.02	—	***	N/A	—	N/A	***	N/A	0.002 ± 0.002*
PHA	0.84 ± 0.01	—	*	N/A	—	2.43 ± 0.39***	—	N/A	—

Figures

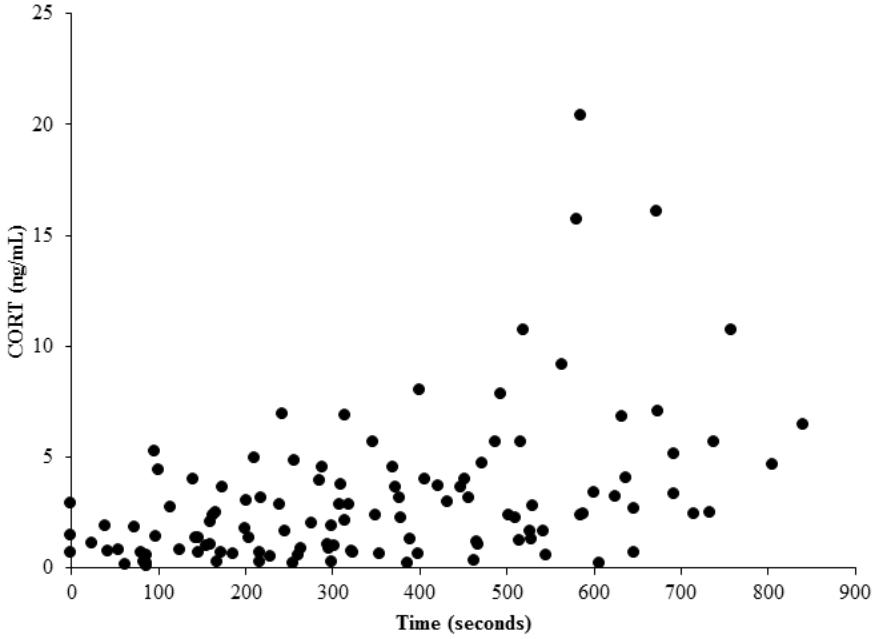

Figure 6.1 The effect of cumulative time (CumTime) to procure a blood sample for CORT analysis.

Although all blood samples were taken within three minutes of capturing an individual, CumTime represents the cumulative time before the sample was taken after entering the pen.

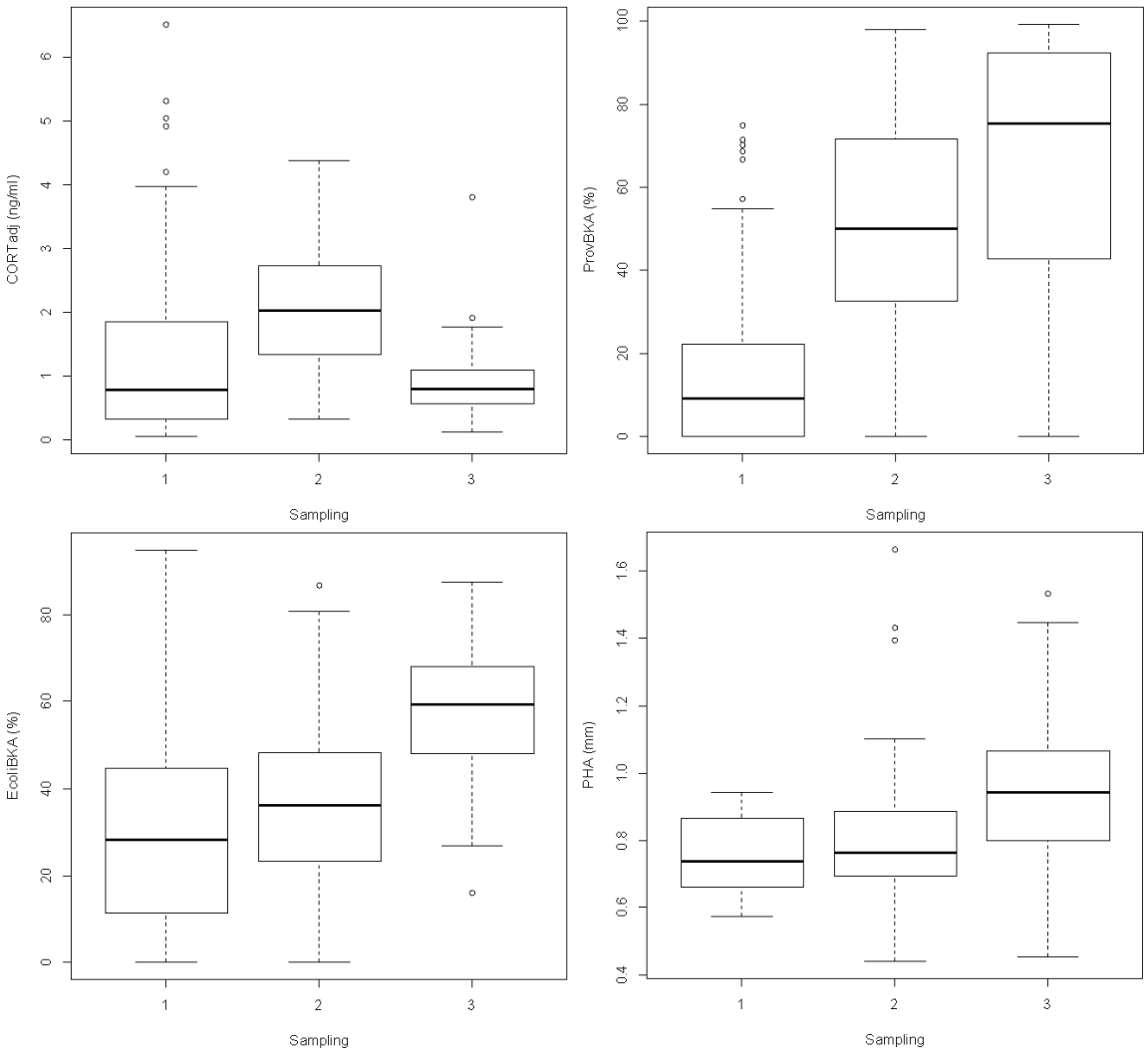

Figure 6.2 Boxplots for time-adjusted plasma corticosterone (CORTadj) and immune traits (ProvBKA, EcoliBKA and PHA) showing medians and heterogeneous variation between sampling periods.

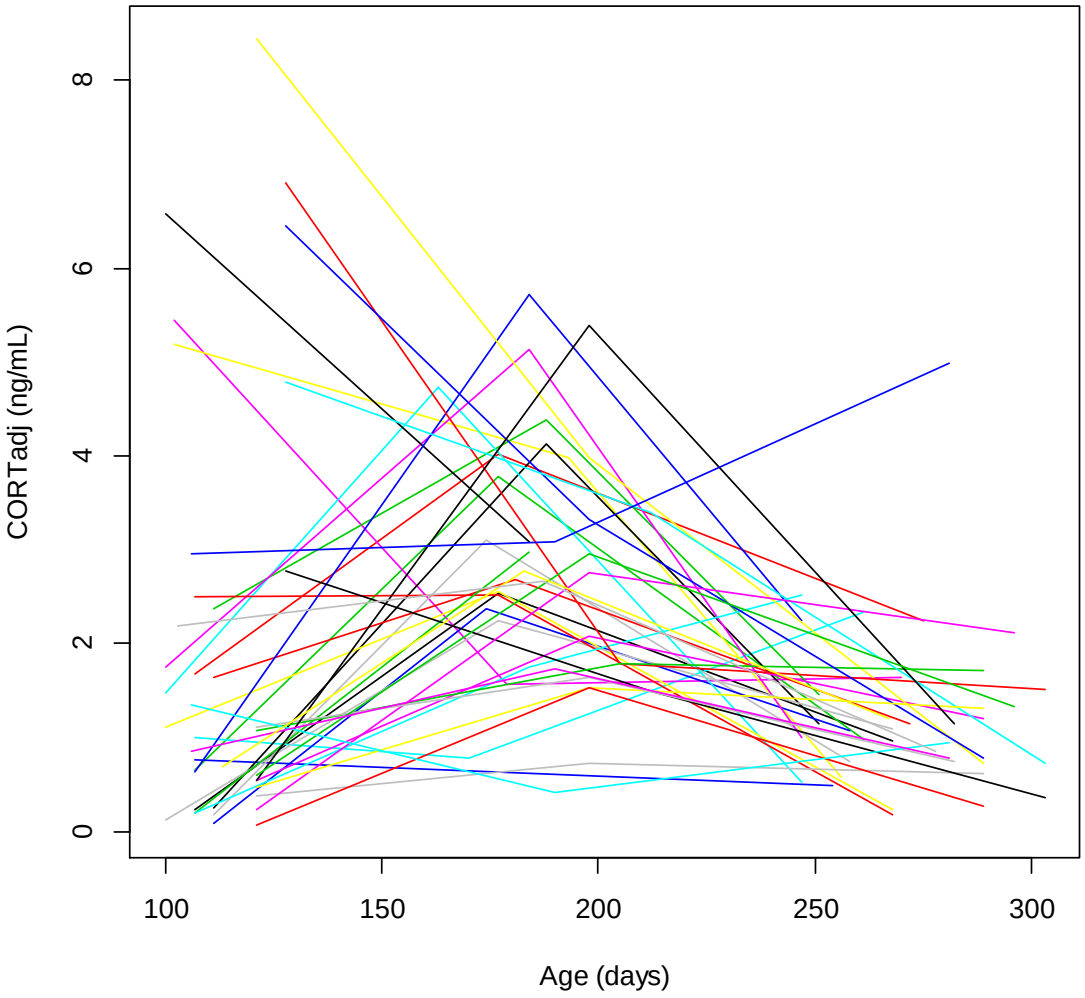

Figure 6.3 Profile of 40 individuals repeatedly sampled for plasma corticosterone. The CORTadj (ng/mL) values have been adjusted for the cumulative time (CumTime) between entering the pen and taking the sample.

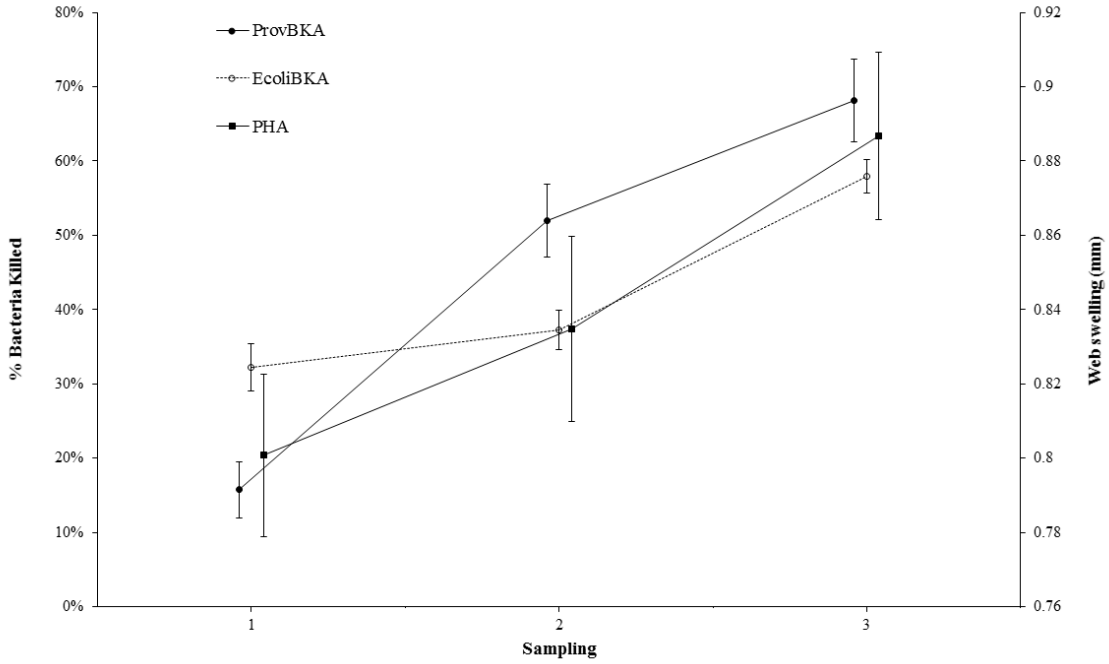

Figure 6.4 Effects of Sampling on ProvBKA, EcoliBKA, and PHA-induced web swelling.

Values are model-based means (± SEM) obtained from univariate models.

CHAPTER 7

CHRONIC INGESTION OF PREY CONTAMINATED WITH COAL COMUBSTION WASTES IN THE AMERICAN ALLIGATOR (*ALLIGATOR MISSISSIPPIENSIS*): UNDERSTANDING IMPACTS ON HEALTH AND IMMUNITY [6]

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[6] Finger, J.W. Jr., M.T. Hamilton, B.S. Metts, R.M. Elsey, T.C. Glenn, and T.D. Tuberville. To be submitted to *Archives of Environmental Contamination and Toxicology*.

Abstract

Coal burning power plants supply approximately 39% of the electricity in the USA. However, incomplete combustion produces ash wastes enriched for toxic trace elements which is often disposed of in aquatic impoundments known as ash basins. Organisms inhabiting such habitats may accumulate trace elements, however, studies investigating the effects on biota have been restricted to shorter-lived, lower-trophic organisms. The American alligator, a long-lived, top trophic carnivore, has been observed inhabiting these basins, yet the biological effects of chronic exposure and possible accumulation remain unknown. In this study, we investigated the immune function and health effects of the feeding of prey contaminated with coal combustion wastes (CCWs) to juvenile alligators for two years. Alligators were grouped into four Feeding groups, including controls and those fed prey contaminated with CCWs for one, two, or three times a week. However, no effect of Feeding Treatment was observed on any immune parameters or plasma analytes, but bacteriacidal ability (p=0.016), splenic mass (p=0.028), and several plasma analytes were influenced by which tanks the animals were housed in. Swelling response following phytohemagglutinin injection was not affected by either Feeding Treatment or the tank an animal was housed in. Our results suggest that exposure to CCWs may not have a negative effect on certain aspects of the immune and hematological system. Future studies are required to further elucidate this.

Introduction

Environmental stressors, such as anthropogenically-derived contaminants, are known to affect a multitude of physiological and behavioral processes (Finger and Gogal 2013; Hopkins et al. 1998; Rowe et al. 2002). One area of concern is exposure to

contaminants through the combustion of fossil fuels for energy production and the subsequent waste produced. In particular, coal burning power plants supply approximately 39% of the electricity consumed in the United States of America (US EIA 2014), however incomplete combustion produces solid waste materials, termed coal comubstions wastes (CCWs) that must be disposed of (NRC 2006). CCWs may be comprised of about 20 trace elements, and depending on source may include copper (Cu), lead (Pb), selenium (Se), mercury (Hg), cadmium (Cd), and arsenic (As; Hopkins et al. 1999; NRC 2006; Rowe et al. 2002). Waste management of CCWs may follow a variety of routes, including disposition in landfills or mines or use in structural products (i.e. cement or concrete; NRC 2006). Residues may also be disposed in artificial aquatic basins termed ash basins, where CCWs and water are amalgamated and subsequently deposited in water-containing impoundments (Rowe et al. 2002; NRC 2006). The ash-water slurry flows into a primary settling basin, then water flows into a secondary basin and is then typically released without further treatment into nearby receiving rivers or other water bodies.

Many types of animals and plants have been documented in or around ash basins (Hopkins et al. 1999; Roe et al. 2004; Rowe et al. 2001; Rowe et al. 2002), which have increased risk of contaminant exposure and consequences. In fact, many of the elements found in ash basins have been shown to accumulate in both plants and animals (Rowe et al. 2002), increasing the risk in higher trophic organisms. However, the effects of exposure CCWshave been mainly investigated in shorter-lived organisms (Hopkins et al. 1998; Hopkins et al. 1999; Hopkins et al. 2002; Rowe et al. 1996; Rowe et al. 2001), with the effects of long-term exposure in longer-lived organisms less well known. In fact, the

majority of studies investigating exposure often utilize organisms of lower trophic status (Hopkins et al. 1998; Hopkins et al. 1999; Hopkins et al. 2002; Rowe et al. 2001), failing to account for how accumulation may affect higher trophic organisms and possibly provide insight into human health effects. Thus, investigations into the effects of CCWs on longer-lived, higher level organisms are necessary for the elucidation of how these compounds may affect organismal and environmental health.

Crocodilians, semi-aquatic archosaurs, have been previously suggested as useful environmental sentinels for contaminant exposure due to their life history (i.e. long-lived) and top trophic placement (Campbell 2003; Milnes and Guillette 2008; Finger and Gogal 2013), allowing for bioaccumulation and biomagnification of compounds. Most studies of contaminants in crocodilians have examined the effects of contaminant exposure on one particular species (Campbell, 2003), the American alligator (*Alligator mississippiensis*), a species endemic to the southeastern United States. However, relatively few studies have investigated the effects of exposure to trace elements present in CCWs (Burger et al. 2002; Campbell et al. 2010). Importantly however, alligators may frequent ash basins, thereby allowing exposure to contaminants through ingestion of water and contaminated prey items (Roe et al. 2004; *personal observation*). Furthermore, previous studies have demonstrated accumulation in other species of vertebrates, some of which alligators may prey upon (Hopkins et al. 1998; Hopkins et al. 1999; Hopkins et al. 2002; Rowe et al. 1996; Rowe et al. 2002). However, it is important to keep in mind that accumulation may be affected by a number of factors, including individual age, sex, or life history (see Campbell et al. 2010). The only study that has investigated the effects of CCW on alligatorsfound increased Se *in ovo* reduced clutch viability in hatchlings and

eggs from a female alligator nesting downstream from a coal burning power plant (Roe et al. 2004), suggesting that accumulation of Se (CCW trace element) and subsequent maternal transfer contributed to these effects. As of yet no study has directly investigated the role of CCWs on crocodilian biology nor have the long-term effects of CCW exposure been elucidated. Therefore, further research is necessary to understand how CCWs may impact a long-lived, top predator.

Herein, we investigate the effects of dietary ingestion and subsequent accumulation of CCWs contaminated prey items on the immune system, health, and size of the American alligator following dietary administration for 2 years. Accumulation of trace elements found in CCWs has previously been shown to increase metabolic rate, however such an increase in metabolic rate is most likely an adaptive response to counteract deleterious effects of contaminants, which consequently may induce trade-offs with other systems, such as immune function or growth (Hopkins et al. 1999; Rowe et al. 2001). Furthermore, exposure to CCWs and the trace elements within the wastes, has previously been shown to deleteriously affect various physiological processes, including endocrine signaling, development, and/or reproduction (Hopkins et al. 1997; Hopkins et al. 2004; Roe et al. 2004), however less is known of how the immune system may be affected. Importantly, however, many of the trace elements present in CCWs have been shown to have deleterious effects on the immune system (Dangleben et al, 2013; Hoffman 2007; Mishra 2009). As the ability of an organism to efficaciously respond following pathogenic challenge (i.e. immunocomptence), is of predominant importance, inability to fend off pathogens may reduce survival and consequently fitness (Demas et al. 2011). In fact, exposure to toxicants and subsequent immune perturbations has been

hypothesized as one of the possible causes of amphibian declines (reviewed by Carey et al. 1999). Therefore, the primary objective of this study was to understand how exposure to CCWs may impact the immune system. A second objective of this study was to investigate the effects of CCWs on body size and health, as measured by blood and plasma analytes.

Methods and Materials

Husbandry and Contaminant Exposure

The pen setups and husbandry protocols, including feeding regimens have previously been described by Tuberville et al. (*in prep*). Briefly, American alligators obtained from Rockefeller Wildlife Refuge in Grand Chenier, LA, USA were transported to the Savannah River Ecology Laboratory on the Savannah River Site (SRS) near Aiken, SC. Thirty-six alligators were randomly distributed among and housed in six tanks in a climate controlled (18-30°C throughout experiment) greenhouse type facility.

Prior to initiation of exposure treatments, alligators were supplemented *ad libitum* with Mazuri crocodilian chow (PMI Nutrition International LLC, Brentwood, MO). For two years animals in each tank were exposed to one of four diet treatments that varied in relative frequency of contaminated vs uncontaminated food offered (Tuberville et al. *in prep*). Animals in all tanks were fed three times per week, but one tank (Tank 1) received contaminated food only once per week (and uncontaminated food the other two feedings); two tanks (Tanks 2A, 2B) received contaminated food twice per week, and two tanks (Tanks 3A, 3B) were fed contaminated prey three times per week. The control tank was only fed uncontaminated food.

Following the capture and blood sampling of all individuals, we measured the snout-vent length (SVL), total length (TL), and tail girth (TG) of each individual as described by Isberg et al. (2005) and Webb and Messel (1978) as indicators of growth. Individuals were also weighed. Alligators were sexed as described by Allsteadt and Lang (1995) by spreading of the cloaca with tweezers.

We had one mortality, a female from Tank 3A that was the smallest individual in the study (SVL 285 mm, TL 460 mm, TG 87 mm, and Mass 324 g). Consequently, this individual was excluded from analysis.

Sample Collection Analysis

Fourteen alligators were originally sampled, euthanized, and organs were removed for analysis 12 months after starting the feeding experiment. However in this study, we will only focus on the remaining 22 alligators that were sampled at the end of a 2-year long feeding experiment. Alligators were captured from respective tanks and blood was obtained from the occipital sinus within 5 minutes of capture for each individual, subsequently inverted, and placed in lithium-heparin tubes on ice.

Prior to centrifugation, heparinized microhematocrit capillary tubes (15401-628; VWR Scientific Inc. Drummond Scientific Co., Broomall, PA, USA) were filled with whole blood from lithium-heparin tubes (as described above) to measure hematocrit (i.e. packed cell volume; PCV). Tubes were then centrifuged for 5 minutes at 10,000 rpm in an LW Scientific ZIPocrit microcentrifuge (Lawrenceville, GA, USA). PCV (in percent) was then determined using the provided ZIPocrit reading chart. Whole blood was also aliquoted for trace element analysis.

Tubes containing whole blood were later centrifuged for one minute at 6,000 rpm. Plasma was aliquoted into 1.5 mL tubes and frozen at -60°C until assays were conducted (described below).

Alligators were euthanized using xylaine (4 mg/kg) and submersion in liquid nitrogen. Alligators were then dissected and organs (kidney, liver, spleen) were removed. Samples from the liver and kidney were stored at -60°C until trace element analysis. The spleen was weighed to the nearest 0.01 gram. We were not able to obtain splenic mass from two individuals (one in Tank 1, one in Tank 3A) and we excluded one splenic value due to only a portion of the organ being weighed (thus, overall n=19).

PHA Injection

Following blood sampling, we injected alligators with PHA (PHA-P #L8754; Sigma-Aldrich, St Louis, MO, USA) using the protocol described by Finger et al. (2013) for saltwater crocodiles (*Crocodylus porosus*) to determine the effects of contaminants on the cellular immune response. As we had previously shown that 2 mg/mL PHA elicited a significant swelling response following injection in saltwater crocodiles, we utilized this same dose to elucidate immune responsiveness in alligators. Briefly, either 20μL of 2 mg/mL PHA or 20μL of sterile phosphate buffered saline (PBS) were injected using a 0.3-mL syringe with a 29-guage needle into the right and left hind toe webs, respectively, between the first and second digits. PBS was used as a control as injection alone is likely to trigger an inflammatory response (Brown et al. 2011; Finger et al. 2013). Web thickness of both webs was measured prior to (0 hr) and 24 hr post-injection using a dial thickness gauge (Peacock G-1A; Ozaki Manufacturing Ltd, Japan), with three rapid,

sequential measurements taken at each time point (Finger et al. 2013). To minimize errors, one person (JWF) performed injections and measurements for standardization. To determine average thickness of each individual at respective time points, the three measurements were averaged (Brown et al. 2011; Finger et al. 2013).

Bacterial Killing Assay

Bacterial killing assays (BKAs) were performed with *Esherichia coli* following the protocol of Finger et al. (Chapter 6 of this dissertation). Briefly, thawed plasma was incubated with CO_2-independent media, subsequently mixed with *E. coli*, and then this mixture was plated onto tryptic-soy agar (TSA) plates. This initial mixing and plating corresponded to time point "0". The mixture was incubated at room temperature for 60 minutes after initial addition of bacteria and then another aliquot was plated out, corresponding to time point "60". Plates were inverted and incubated at 37°C for 24 hours and colonies were enumerated. Calculations for percent bacterial killed have previously been described (Chapter 6 of this dissertation).

Hematology and Biochemistry

On the same day we ran BKAs, frozen plasma was analyzed using an Abaxis VetScan VS2 (Abaxis North America, Union City, CA, USA). Individual plasma samples were loaded into cartridges specific for the Avian/Reptile profile. Analytes measured included aspartate aminotransferase (AST), bile acids (BA), uric acid (UA), total proteins (TP), albumins (ALB), globulins (GLB), glucose (GLU), phosphorous (Ph), creatine kinase (CK), calcium (Ca), potassium (K^+), and sodium (Na^+).

Trace Element Analysis

Analysis of trace elements have been described by Tuberville et al. (*in prep*). In this study, trace element concentrations in the kidney and liver, 2 years after initial dosing, were used to assess effects on the immune and hematological systems. All concentrations in the liver and kidney were determined to the nearest mg/kg (i.e. parts per million; ppm). For elemental values below the median detection limit (<MDL), we took half the calculated detection limit to provide values for these metals. Liver Tl was <MDL for three individuals and kidney Ni was <MDL for two individuals. Almost half of all individuals displayed values of liver Sb and liver Ni that were <MDL, therefore these elements were excluded from statistical analysis.

Statistics

Due to unequal sample sizes, an unbalanced analysis of variance (ANOVA) using a generalized linear model (GLM) with Feeding Treatment (0, 1, 2, or 3 times per week) or Tank as factors was performed to test for effects on mean morphometric indices. When significant, SVL was used as a covariate in subsequent analyses (detailed below).

Simple regression was used to investigate the effect of Feeding Treatment as a discrete dosage factor to determine if Feeding Treatment had any impact on concentrations (parts per million; ppm) of trace elements in the liver and kidney. Residuals from univariate models of trace elements significantly affected by Feeding Treatment were used as covariates in subsequent analyses as detailed below (i.e. Feeding Treatment adjusted residuals; see Chapter 6). Simple regression was also used prior to more sophisticated modelling to determine how overall individual trace elements that were significantly affected by the ingestion of prey contaminated with CCWs (i.e.

Feeding Treatment adjusted residuals), as detailed above, affected hematological, biochemical, and immunological indices irrespective of size, the tank an animal was housed in, or the Feeding Treatment (i.e. neither Tank nor Feeding Treatment used as factors; no covariates).

Due to repeated measurements on the same individual and to understand the validity of the assay irrespective of Tank or Feeding Treatment, a linear mixed model (LMM) using restricted maximum likelihood (REML) was used to evaluate the effect of the factors Time (0 vs 24 hrs) and Treatment (PHA vs. PBS) on Web Thickness following injection (Finger et al. 2013). AnimalID was used as a random effect to account for repeated measures (i.e. Time 0, 24 hrs) of Web Thickness at PBS and PHA injection sites on the same individual (Finger et al., 2013). To understand the effects of Feeding Treatment or Tank on the difference in PHA and PBS induced Swelling 24 hrs post injection (i.e. PHA24 minus PBS24), indicated by the term "Swelling" below, we used a GLM (i.e. multiple regression).

Due to the lack of repeated measures and the unequal sample sizes between groups, GLMs (i.e. multiple regression) were used to analyze percent bacteria killed, Swelling (as described above), hematological and biochemical indices, and splenic mass. AST, PCV, and Ph were natural log transformed to achieve normality (i.e. LnAST, LnCK, LnPCV, and LnPh). Factors in analysis consisted of Feeding Treatment (0, 1, 2, or 3 times/week as detailed above) and Tank (the tank of origin; n=6). Feeding Treatment, hereafter referred to as "Feeding", and Tank were not used simultaneously as factors in analysis because they were confounded (i.e. specific tanks were fed a certain amount of times per week). However, the terms were not completely confounded within one another

due to unequal numbers of Feeding groups (i.e. Tanks 3A and 3B fed thrice weekly, Tanks 2A and 2B fed twice weekly). Therefore, we used both Tank and Feeding as factors in respective analyses to differentiate Tank effects from Feeding effects. When both Tank and Feeding were respectively significant, we used the term with the lower *p*-value in the final model.

Covariates in analysis of Swelling, BKAs, hematological and biochemical indices, and splenic mass included SVL and trace element (Feeding-adjusted residuals) concentrations (ppm) when significant. The Handling Time between capture and obtaining a blood samples (in seconds) was used as a covariate for BKAs and hematological and biochemical data when significant. Sex was not used as a factor in analysis due the disproportionate number of females (n=18) to males (n=3).

Results are presented as means ± standard errors (SE). If data were initially log transformed, then means represent predicited back-transformed values. Values for a particular variable were removed if residuals were greater than four standard deviations from the mean of the residual. Least significant differences (LSDs) at the 5% level (i.e. 5% LSD) were used to compare means in post-hoc analysis. As described above, one individual was removed from analysis due to morality. All analyses were performed in GenStat Version 16.1(VSN International Ltd, Hemel Hempstead, UK). A value of $p<0.05$ was considered significant.

Results

Morphometrics

Raw morphometric means for Tank, Feeding, and Sex are shown in Table 7.1. We observed no effect of Feeding or Tank on SVL, TL, TG, or Mass, but post-hoc analysis

revealed Feeding group and Tank differences in SVL. Control individuals had a significantly longer SVL (369.7 ± 14.38 mm) than individuals fed three (333.1 ± 9.42 mm) times a week (5% LSD). Individuals in Tank 2B (367.2 ± 12.71) had a significantly longer SVL than those in Tank 3A (325.7 ± 14.67 mm). Therefore, we used SVL as a covariate when significant to account for variation between Feeding groups and/or Tanks.

Feeding Effects on Trace Element Concentrations

Concentrations of trace elements in the liver and kidney and accumulation over a 2-year period are detailed in Tuberville et al. (*in prep*). In this study, to evaluate the effectiveness of Feeding as a discrete dosage factor affecting immunological and hematological parameters, we performed simple regression looking at the effect of Feeding (using SVL as a covariate) on trace element concentrations in the kidney and liver. Feeding had no effect on levels of Cu, Ni, Sb, Pb, Zn, and Cr in the kidney or levels of V, Cr, Cu, and Zn in the liver (Table 7.2).

However, Feeding had a significant effect on Cd ($p<0.001$; KidCd), Se ($p<0.001$; KidSe), and Tl ($p<0.001$; KidTl) levels in the kidney. Significant differences were observed at each Feeding level, with individuals fed more frequently exhibiting increased amounts of each respective trace element (5% LSD). Levels of Sr in the kidney ($p<0.001$; KidSr) and levels of As (LivAs) and Se (LivSe) in the liver were significantly affected by Feeding (LivAs $p<0.001$; LivSe $p<0.001$) and SVL (LivAs $p=0.009$; LivSe $p=0.03$), but controls for each of these trace elements were not significantly different from those fed once a week (5% LSD). Feeding had a significant effect on liver levels of Tl ($p<0.001$; LivTl). Both Feeding ($p<0.001$) and SVL ($p=0.005$) significantly affected kidney As (KidAs) levels. Interestingly, however there were no significant differences in either

LivTl or KidAs between individuals fed two and three times weekly (5% LSD). Feeding also significantly affected liver Sr (p=0.001; LivSr) and kidney V (p=0.004; KidV), however there were no differences between groups fed two and three times a week and between control and individuals fed once a week (5% LSD). Liver levels of Cd were significantly affected by Feeding (p<0.001; LivCd), however no differences were found between individuals fed one and two times weekly (5% LSD). Although Feeding had a significant effect on liver Pb levels (p=0.006; LivPb), there were no significant differences between the control group and any groups fed FAC prey (5% LSD).

The residuals from univariate models examining the effect of Feeding on trace element concentrations were used as covariates in subsequent analyses to adjust for the effect of Feeding on a particular trace element of interest. All effects and values of trace elements presented hereafter represent the Feeding-adjusted, along with SVL-adjusted when significant, residuals from respective univariate models.

Effects of Individual Trace Element Concentrations on Blood and Immune Parameters

Irrespective of Feeding, size, or Tank, we performed simple regression to understand the effects of individual trace element concentrations on blood and immune parameters. The output (i.e. *p* values) from simple regression models is shown in Table 7.3. Subsequently, individual elements were then incorporated into more sophisticated models to better elucidate other factors influencing hematological, biochemical, and immunological indices as detailed below.

PHA Injection

We analyzed the effect of Time (0 vs. 24 hrs) and Treatment (PHA vs. PBS) on the swelling response (i.e. Web Thickness) independent of Feeding or Tank to evaluate the successful (i.e. swelling greater in PHA injected) implementation of the technique. We observed a significant Time×Treatment interaction ($p<0.001$) on Web Thickness (Figure 7.1), with Web Thickness at PHA24 (1.13 ± 0.02 mm) significantly greater than Swelling at PBS24 (0.76 ± 0.02 mm) and PHA0 (0.73 ± 0.02 mm; 5% LSD). Such an enhanced response relative to PBS is expected, as PHA is a polyclonal antigenic lectin triggering inflammation and leukocytic extravasation following injection (Finger et al., 2013). Web Thickness at PBS24 was also significantly greater than at PBS0 (0.70 ± 0.02 mm), demonstrating that the action of injection alone triggers a response (5% LSD). There were no differences in Web Thickness prior to injection in either toe (PHA0 vs. PBS0; 5% LSD).

Feeding, nor Tank, had no effect on the difference in Swelling (PHA minus PBS) 24 hours after injection (Figure 7.2). Difference in Swelling 24 hours after injection in control and those fed once, twice, or thrice weekly were 0.41 ± 0.04 mm, 0.33 ± 0.12 mm, 0.31 ± 0.04 mm, and 0.48 ± 0.07 mm, respectively.

Bacterial Killing Assays

BKAs with *E. coli* were significantly affected by Tank ($p=0.016$; Figure 7.3). Individuals in Tank 3A had the highest bactericidal capabilities (89.47 ± 0.06 %). Individuals in Tanks 3A, 2B (88.37 ± 0.04 %), 3B (88.86 ± 0.04 %), and 1 (85.97 ± 0.04 %) all exhibited significantly enhanced killing ability relative to Tank 2A (64.34 ± 0.05 %; 5% LSD). Predicted means for the control tank were 75.27 ± 0.05 %.

Splenic Mass

Splenic mass was significantly affected by Tank (p=0.028) and KidSe (p=0.008). The splenic mass of individuals in Tanks 3A (1.24 ± 0.16), 3B (0.85 ± 0.11), 2A (1.04 ± 0.12), and control (1.07 ± 0.16) were significantly greater than those in Tank 1 (0.40 ± 0.13). Splenic mass of individuals from Tank 3A was also significantly greater than those from Tank 2B (0.82 ± 0.11). Levels of KidSe had a negative effect on splenic mass (0.22 ± 0.07).

Hematological and Biochemical Parameters

CK was significantly affected by Tank (p=0.011), Handling Time (p<0.001), and LivSr (p=0.044). Individuals from Tank 2B (1370.8 ± 287.2 U/L) had significantly greater CK than individuals from Tank 3B (362.2 ± 278.5 U/L) and 1 (325.1 ± 277 U/L; 5% LSD). Levels of CK from individuals from the control tank (2074.4 ± 333.4 U/L) were significantly greater than those in Tanks 2A (566.6 ± 337.1 U/L), 3B, and 1 (5% LSD). Levels of CK in Tank 3A were 1074.6 ± 401.4 U/L. Both Handling Time (23.02 ± 2.87 U/L) and Liv_Sr (306.8 ± 136.07 U/L) increased CK levels.

Plasma Ph was significantly impacted by Tank (p<0.001), KidCd (p=0.008), and LivTl (p=0.039). Levels of Ph were significantly elevated in individuals from Tank 2B (6.39 ± 0.39 mg/dL) relative to individuals in Tank 3B (4.66 ± 0.27 mg/dL) and 1 (4.96 ± 0.28 mg/dL). Levels of Ph in Tanks 2A, 3A, and the control tank were 5.55 ± 0.39 mg/dL, 5.82 ± 0.49 mg/dL, and 5.39 ± 0.36 mg/dL, respectively. Levels of both KidCd (1.10 ± 0.03) and LivTl (1.07 ± 0.03) had a negative effect on plasma Ph.

Plasma K^+ was significantly affected by Tank (p=0.029). Levels of K^+ in individuals from Tank 3A (5.14 ± 0.30 mmol/L) were significantly higher than those in

Tanks 2B (4.09 ± 0.17 mmol/L), 3B (4.31 ± 0.18 mmol/L), 1 (4.07 ± 0.17 mmol/L), and control (4.25 ± 0.20 mmol/L; 5% LSD). Levels were significantly greater in Tank 2A (4.78 ± 0.22 mmol/L) relative to Tank 2B and Tank 1 (5% LSD).

Tank had a significant impact on PCV (p<0.001). Individuals in Tanks 2B (33.45 ± 8.11 %), 3B (26.21 ± 5.50 %), and 1 (30.57 ± 6.42 %) displayed PCV values that were significantly greater than those in Tanks 3A (10.49 ± 2.54 %) and 2A (5.77 ± 1.40 %). PCV values in the control tank (17.76 ± 4.31 %) were significantly greater than those in Tank 2A.

No effects of the factors Tank or Feeding were observed on AST, GLU, Ca^{2+}, TP, and Na^{+} (Table 7.4). Simple regression performed to understand impacts of trace element concentrations on all hematological parameters is shown in Table 7.3. Values of all plasma analytes and PCV are displayed in Table 7.4.

All individuals displayed values of BAs and ALBs that were lower than the limit of detection (LOD). One individual had UA levels above LOD (Tank 3A), however all other individuals displayed values less than LOD. Chemistry suppression due to interference within samples prevented obtainment of GLB values (calculated from total ALBs and TS). Therefore, these (BAs, ALBs, and GLB) data are not shown. We were also unable to quantify CK values for one individual (Tank 3A).

Discussion

Expectedly, chronic Feeding of prey contaminated with CCWs had a significant effect on final concentrations of most trace elements in the liver and kidney. As detailed in Tuberville et al. (*in prep*), individuals accumulated amounts of trace elements over the two-year period. However, as to why some trace element concentrations were not

affected by Feeding is likely related to the prey items themselves being relatively lower in concentration for those trace elements (Tuberville et al. *in prep*), the fact that simple regression may not have been sensitive enough to dissociate out any differences in Feeding regimes, or that specific elements may not accumulate in the kidney and/or liver.

Body Size

Due to previous studies suggesting that FAC exposure may inhibit or reduce growth due to trade-offs (Hopkins et al. 1999; Rowe et al. 2001), one objective of this study was to determine the effect of chronic ingestion of CCWs on the size of an individual. Herein we analyzed four measurements of size including HL, SVL, TL, and Mass, observing no effects of Feeding or Tank on any morphometric parameter. These results suggest that ingestion of CCWs for two years had no effect on size. Similar to our results, Hopkins et al. (2002) also observed normal body growth and condition (relative to controls) in juvenile banded water snakes (*Nerodia fasciata*) following a two yearlong feeding of CCWs even though snakes accumulated significant amounts of trace elements. In the only other study examining the effects of CCWs on crocodilians, Roe et al. (2004) investigated how hatchling alligators from an ash basin and those from CCW contaminated downstream site were impacted by CCW exposure relative to an unpolluted control site. Whilst Roe et al. (2004) described differences in raw mass, SVL, and TL, this was not supported with any statistical tests, therefore we are unable to determine how or if CCWs present in ash basins had any significant impacts on the growth of hatchlings in that particular study. In our study, however, when we conducted LSDs to determine differences between group means, we found that control individuals were significantly longer (SVL) than those fed three times a week. This result may suggest that the

ingestion of CCWs may negatively affect individual size or grwoth, but that these effects may only be observed at higher concentrations. Further research should aim to investigate this more fully.

In captive settings, such as farms, crocodilians are often graded (i.e. similar sized individuals are moved into pens together) continuously to prevent variations in size from occurring and larger individuals monopolizing resources (Morpurgo et al. 1993; Chapter 6 of this thesis). However, in this study, grading was not feasible due to differences in Feeding regimens, Tank setups within those Feeding regimens, and a finite amount of space for housing alligators. As such, the factor Tank was incorporated into analysis to investigate such effects of non-grading and the individual environments of each respective pen. The result that Tank had no impact on any morphometrics (save for the post-hoc result) suggests that any differences attributed to growth would have been brought about by the different feeding regimens and/or trace element concentrations.

The Effects of Feeding on The Immune System

Due to previous studies suggesting that the trace elements present in CCWs may suppress immune function (e.g. Dangleben et al. 2013; Fairbrother and Fowles 1990; Mishra 2009; however see Fair and Ricklefs 2002), another objective of this study was to determine the effects of feeding CCWs on the immune system. However, our results suggest that exposure to and accumulation of CCWs does not detrimentally affect the immune system.

Injection with PHA, a non-pathogenic, polyclonal antigenic lectin, has been used in a multitude of studies as a routine measure of an individual's ability to mount an immune response which is manifested macroscopically by an increase in swelling

following injection (usually 24 hours after injection; Brown et al. 2011; Finger et al. 2013). Primary injections, as were conducted in this study, measure the innate, inflammatory potential whereas subsequent injections may provide insight into specific, acquired immunity to repeated exposure to the same antigen (Brown et al. 2011; Finger et al. 2013). Expectedly, at 24 hrs post-injection, all individuals in our study (regardless of Tank, Sex, or Feeding) exhibited a significantly increased swelling response following PHA injection relative to PBS injection, a finding we have previously observed in saltwater crocodiles (Finger et al. 2013).

The null effect of Feeding on the difference in Swelling (PHA minus PBS) 24 hours after injection reflects that ingestion of CCW prey and subsequent accumulation of trace elements does not inhibit the responsiveness to novel antigens, such as PHA. This result suggests that exposure to CCWs may not affect the ability of an organism to mount an immune response. Following invasion, pathogens are recognized and ingested by innate cells (i.e. phagocytes; Abbas et al. 2010). These innate cells, such as dendritic cells, subsequently present degraded pathogenic antigens to lymphocytes, the mediators of the adaptive immune system, which consequently facilitate a number of responses aimed at microbial elimination (Abbas et al. 2010).

Our results illustrate that bactericidal capacity was not affected by CCWs, suggesting that chronic ingestion of CCWs may not impact constitutive innate defenses present in the plasma. Complement proteins (CPs), which are an essential component in innate defense against microbes present in plasma (Finger and Isberg 2012), are primarily produced by hepatocytes, however, other types of cells may also produce CPs, such as fibroblasts and epithelial cells (Morgan and Gasque 1997; Qin and Gao 2006). Alligators

fed CCWs prey accumulated substantial amounts of trace elements (Tuberville et al. *in prep*), therefore a logical postulation would be that this accumulation may have perturbed protein production and consequently affected plasma bactericidal capabilities. However, this was not observed.

The bacterial killing ability of an individual was influenced by Tank of origin. The BKA measures constitutive immune mediators present in the plasma, including antimicrobial proteins and peptides, antibodies, and complement proteins (Chapter 6; Demas et al. 2011), with killing ability related to the decrease in the number of colonies after incubation with an individual's plasma. A possible factor influencing immune function in ectothermic crocodilians is temperature and indeed, previous studies have shown that temperature influences plasma microbicidal activity (reviewed by Finger and Isberg 2012). Therefore, logically, one possible explanation for differences in Tank killing ability would be a difference in temperatures between Tanks. However because all pens in this study were provided with ambient heat via heat lamps, water from the same sources, and were housed in a climate controlled-greenhouse type facility (Tuberville et al., *in prep*), therefore it seems unlikely that differences were attributable to temperature deviations between the Tanks (Finger and Isberg 2012; Zimmerman et al. 2010a). Furthermore, whilst other studies have suggested that bactericidal ability may increase with age or size of an individual (Zimmerman et al. 2010b), all individuals in this study were of a similar age and size (see above). However, in spite of all these controls, we were still unable to completely standardize across all tanks as observed with the significant effect of Tank on BKA. These Tank differences may also be related to

differences in disease status or susceptibility between and within Tank-mates, however this has yet to be investigated.

The spleen is a peripheral immune organ that aids in blood filtration, pathogen-immune interactions, and removal of senescent erythrocytes (Abbas et al. 2010; Finger and Isberg 2012). In fact, since crocodilians do not appear to possess lymph nodes nor germinal centers (Finger and Isberg, 2012), the importance of the spleen in pathogenic defense may be paramount (Rooney et al. 2003). As a routine immunotoxicological test, we weighed the spleen to examine how CCW exposure may affect splenic mass, and consequently its function. For example, changes in organ mass, such as those that may manifest as organ hypertrophy or atrophy, may reflect toxicity (see Sellers et al. 2007). Although organ mass may often be used in conjunction with histological studies to better evaluate how toxicants may affect tissues (Haley et al. 2005; Sellers et al. 2007), we were unable to perform histology on the spleen due to budgetary and time constraints.

Feeding had no effect on splenic mass. However, there was a significant effect of Tank. Although these results suggest that chronic ingestion and accumulation of CCWs does not affect alligator splenic mass, histological examination following administration of CCWs would provide further evidence of the impact (or lack thereof) of CCWs on splenic function.

Hematology and Blood Biochemistry

Hematology and blood biochemistry were analyzed to better understand how CCWs exposure may affect health (Table 7.4). Our results indicate that administration of CCWs had no impact on any plasma analytes or PCV. However, levels of CK, K^+, Ph, and PCV were significantly impacted by the tank an individual was housed in.

Ranges for hematological and biochemical parameters have been previously provided for other crocodilian species (Lovely et al. 2012; Zayas et al. 2011), including the American alligator (Barnett et al. 1998; Dessauer; 1970; Guillette et al. 1997; Lance et al. 1983; Lance and Elsey 1999). However, it is important to emphasize that many variables can affect these values, including size, habitat, the type of prey ingested, season, or reproductive status (Guillette et al. 1997; Lance et al. 1983; Lance and Elsey 1999; Lovely et al., 2012; Zayas et al. 2011). A summary of previously provided parameters pertinent to this study in alligators are summarized in Table 7.5.

As evident from Table 7.5, the values obtained in this study do not appear drastically different from those in previous studies, suggesting that exposure to CCWs through dietary ingestion did not affect alligator health. However, AST levels appear quite high relative to previous provided levels. Also, we were unable to locate previously provided levels of CK in alligators.

Interpretational Caveats and Confounders

Whilst the results provided in this study suggest that chronic ingestion of prey contaminated with CCWs may not affect health or the immune system of the American alligator, our observations may have been impacted by other, unforeseen factors. For example, although alligators were housed in a controlled facility (Tuberville et al. *in prep*), alligators did not feed as often during the colder months, which may have impacted bioaccumulation of CCWs. Moreover, the social interactions of Tank cohabitants may have masked interpretation of the impact of CCWs on the immune system, size, or blood parameters. In fact, crocodilians are known to establish hierarchies (Brien et al. 2013), and in many controlled settings larger individuals are removed from

smaller cohorts to facilitate growth (Elsey et al. 1990). These social interactions, along with other unaccounted for Tank-specific environmental variations, probably contributed to the observed Tank effects (see Chapter 6). It is important to emphasize that individual crocodilians may respond differently to the environment, even when exposed to identical conditions. For example, runt *C. porosus* that fail to grow relative to other clutch members even when raised in the same conditions (Isberg et al. 2009). These runts exhibit increased corticosterone (Isberg et al. 2009; Shilton et al. 2014), the main reptilian stress hormone, relative to 'normal' growing individuals. Although our study lacked runts, any observed Tank effects may be a reflection of specific Tank stressors that may have confused our interpretation of the effects of CCWs on growth or blood and immune parameters. Another challenge of studying long-lived organisms such as alligators, is the amount of time necessary for detrimental immune effects to manifest. For example, feeding of only two years may not be 'enough' time to allow for the detrimental effects of CCWs to manifest as alligators are long-lived and slow maturing reptiles possibly living up to around 50 years (see Woodward et al. 1995).

Another interpretational caveat is the fact that immunological techniques utilized in this study may not have been sensitive enough or may not have measured the correct parameter affected by exposure. Although a multitude of techniques have been developed to assist in determination of organismal immune function (reviewed by Demas et al. 2011), each of these techniques have their own limitations and as such, cautionary analysis is required when examining each technique. For example, PHA injection may be affected by whole host of variables, including size, environment, reproductive status, nutrition, and/or age (see Finger et al. 2013). Furthermore, increased swelling response to

PHA may not be indicative of an organism's resistance to pathogens (e.g. Saks et al. 2006). It is also important to emphasize that pathogenic infection or stress may also affect immunoassays, such as complete blood counts (Davis et al. 2008; Glassman et al. 1979). Moreover, increased splenic mass may also be associated with infection or changes in seasons, with the latter often observed in ectotherms (Zimmerman et al. 2010a). Due to these ambiguous interpretations of what exactly constitutes an effective immune response in a particular immune assay, we employed a number of techniques to better ascertain how CCWs may affect the immune system. However, even though we used a panel of techniques, these techniques may still not have accounted for all impacts on the immune system. In fact, some of the techniques used herein have been recently criticized for their apparent lack of sensitivity (Finger et al. *in prep*, Chapter 6). Unfortunately, however, due to time constraints we were unable to incorporate novel assays into this experiment. We were also unable to conduct histopathology studies on the spleen, which has been suggested to be used in conjunction with organ mass to identify toxic effects on immune system (Haley et al. 2005; Sellers et al. 2007).

One potential limitation of this study was the lack of repeated measurements on individuals (Finger et al. *in prep*, Chapter 6). Taking only one measurement may have allowed for confounding variables, unbeknownst to us, to preclude accurate elucidation of the effects of contaminant exposure on the particular health parameter of study. As described above, many of these measured hematological, biochemical, and immunological parameters or assays may be affected by a whole host of extrinsic variables (Finger and Isberg 2012; Franklin et al. 2003; Glassman et al. 1979; Sykes and Klaphake 2008). We attempted to minimize environmental stressors, such as climate or

circadian rhythms, by housing similar-sized individuals in Tanks within the same climate controlled building, providing each Tank with heat lamps, setting 12 hour light:dark cycles within each tank, and maintaining water temperature within each Tank at 25°C (Tuberville et al. *in prep*). Furthermore, in attempt to prevent increased corticosterone and subsequent perturbations in hematological or immunological parameters, we obtained blood within 3-5 minutes of capture (Romero and Reed 2005). However, our mere presence inside the pen housing the animals may have acted as unaccounted for stressor (Finger et al. *in prep*, Chapter 6).

As stated above, Sex was not used as a factor in analysis due to the disproportionate number of females relative to males. Alligators, like all crocodilians, exhibit temperature-dependent sex determination, with even small (± 1°C) deviations influencing sex (Lang and Andrews 1994). Incubation temperature for alligators in this study ranged from 29-31.8°C, temperatures where females usually predominate (Lang and Andrews 1994), therefore the disproportionate number of females following random sampling was likely unavoidable. As females and males may display sex-specific differences in response to exposure CCWs (e.g. Hopkins et al. 2002), future studies should investigate this.

Conclusions

In conjunction with the study by Tuberville et al. (*in prep*), this is one of the first studies to investigate how trace elements present in CCWs may affect the health of the American alligator, a long-lived, top trophic carnivore. Our results suggest that chronic ingestion of prey contaminated with CCWs does not detrimentally affect the immune system or the health of alligators. However, our results also highlight some apparent

questions and concerns regarding the observed inter-Tank differences that could not be accounted for. These results also necessitate further studies to better understand the mechanisms of how ingestion of prey contaminated with CCWs may contribute to immune or hematological perturbation. Ensuing studies should be undertaken with the implementation of novel techniques and repeated samplings to further elucidate the effect of trace elements in CCWs on the health and immune system of a long-lived, top trophic carnivore.

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Tables

Table 7.1 Raw mean (± standard error) snout-vent length (SVL), total length (TL), tail girth (TG), and weight for each Tank, Feeding group, and Sex.

The associated letter with Tank indicates each individual tank (n=6 tanks). Feeding indicates number of times individuals within a tank were fed per week (0, 1, 2, or 3). Numbers of individuals in each respective Feeding Treatment, Tank, or Sex are indicated below with an *n*.

		SVL (mm)	TG (mm)	TL (mm)	Mass (g)
Tank	CONTROL *n*=3	369.7 ± 6.23	133 ± 4.73	730.3 ± 6.49	982 ± 48
	1 *n*=4	333 ± 6.10	115.2 ± 3.61	654.2 ± 14.10	700 ± 52.13
	2A *n*=3	349 ± 15.95	130.7 ± 4.70	693 ± 26.50	898.7 ± 132.3
	2B *n*=4	367.2 ± 20.76	123.8 ± 11.06	671.5 ± 50.66	853.5 ± 190.4
	3A *n*=3	325.7 ± 8.09	119 ± 4.36	635.7 ± 9.24	712 ± 66.61
	3B *n*=4	338.8 ± 12.64	125.8 ± 3.45	669.8 ± 25.14	764 ± 111.4
Feeding Treatment	0/week *n*=3	369.7 ± 6.23	133 ± 4.73	730.3 ± 6.49	982 ± 48
	1/week *n*=4	333 ± 6.10	115.2 ± 3.61	654.2 ± 14.10	700 ± 52.13
	2/week *n*=7	359.4 ± 13.16	126.7 ± 6.33	680.7 ± 29.20	872.9 ± 113.8
	3/week *n*=7	333.1 ± 7.88	122.9 ± 2.82	655.1 ± 15.50	741.7 ± 65.52
Sex	F *n*=18	347.2 ± 6.38	126.3 ± 2.60	683.8 ± 12.13	844.3 ± 50.88
	M *n*=3	346.7 ± 21.84	111 ± 6.35	617 ± 31.50	616.7 ± 83.72

F=female, M=male

Table 7.2 Concentrations of trace elements in the liver and kidney that were not affected by the Feeding of Coal Combustion Wastes (CCWs).

Control, 1, 2, or 3, indicate number of times fed per week. Mean (±SE) concentrations for respective trace elements are provided in parts per million (ppm)

			Feeding CCWs/Week		
		Control	**1**	**2**	**3**
Trace Element Concentrations (ppm)					
Liver	Cu	48.52 ± 12.11	25.72 ± 3.51	28.72 ± 4.14	37.16 ± 4.46
	Cr	1.42 ± 0.08	1.38 ± 0.10	1.45 ± 0.08	1.36 ± 0.07
	V	1.78 ± 0.18	1.54 ± 0.09	1.69 ± 0.11	1.92 ± 0.21
	Zn	64.77 ± 2.90	54.41 ± 2.90	53.75 ± 3.95	57.36 ± 3.49
Kidney	Cu	8.76 ± 0.39	9.37 ± 0.39	9.68 ± 0.35	10.31 ± 0.74
	Cr	1.67 ± 0.09	2.27 ± 0.73	1.54 ± 0.11	1.43 ± 0.08
	Ni	0.35 ± 0.03	0.54 ± 0.22	0.49 ± 0.08	0.72 ± 0.06
	Pb	0.19 ± 0.05	0.20 ± 0.02	0.21 ± 0.03	0.35 ± 0.08
	Sb	.32 ± 0.06	0.19 ± 0.04	0.16 ± 0.02	0.21 ± 0.03
	Zn	77.43 ± 6.57	76.94 ± 3.65	79.4 ± 2.72	76.19 ± 2.02

Table 7.3 Effect of individual trace element concentrations in the liver and kidney on individual immunological, biochemical and hematological indices.

Significant values (p<0.05) are bolded. Values that were ≥0.05 are indicated with an "—".

Trace Element	BKA	Swelling Diff	K^+	PCV	AST	CK	GLU	Ca^{2+}	Ph	TP	Na^+	Spleen Mass
Liver As	–	–	–	–	–	–	–	–	–	–	–	–
Liver Se	**0.045**	–	–	–	–	–	–	–	–	–	–	–
Liver Sr	–	–	–	–	–	–	–	–	**0.038**	–	–	–
Liver Cd	–	–	–	–	–	–	–	–	–	–	–	–
Liver Pb	–	–	–	–	–	–	–	–	–	–	–	–
Liver Tl	–	–	–	–	–	–	–	–	–	–	–	–
Kidney V	–	–	–	–	**0.004**	–	–	–	**0.019**	–	–	–
Kidney As	–	–	–	–	–	**0.022**	–	–	–	–	–	–
Kidney Se	–	–	–	–	**0.026**	**0.013**	–	–	–	–	–	–
Kidney Sr	–	–	–	–	–	–	–	**0.036**	–	–	–	–
Kidney Cd	–	–	–	–	–	–	–	–	–	–	–	–
Kidney Tl	–	**0.002**	–	–	–	–	–	–	–	–	–	–

BKA=bacterial killing assay; Swelling Diff=Difference in Swelling (PHA minus PBS) 24 hours post injection; AST=aspartate aminotransferase; CK=creatine kinase; GLU=glucose; Ca^{2+}=total calcium; Ph=phosphorus; TP=total proteins; K^+=potassium; Na^+=sodium

Table 7.4 Hematological and blood biochemical parameters of respective Tanks. Column headings are bolded and indicate respective parameters. Units are displayed below each parameter. Asterisks (*) located next to parenthesized units indicate predicted means (±SE) provided from univariate regression models. All other means (±SE) are from raw data. Rows indicate respective Tanks.

		AST (U/L)	**CK (U/L)***	**GLU (mg/dL)**	**Ca^{2+} (mg/dL)**	**Ph (mg/dL)***	**TP (g/dL)**	**K^{+} (mmol/L)***	**Na^{+} (mmol/L)**	**PCV (%)***
Tank	**3A**	217.5 ± 3.5	1074.6 ± 401.4	71.5 ± 4.50	10.4 ± 0.20	5.39 ± 0.36	3.9 ± 0.10	5.14 ± 0.30	138.5 ± 0.50	10.49 ± 2.54
	3B	276 ± 23.77	362.2 ± 278.5	62.5 ± 2.90	10.28 ± 0.36	4.66 ± 0.27	4.1 ± 0.18	4.31 ± 0.18	138 ± 1.23	26.21 ± 5.50
	2A	242 ± 16.2	566.6 ± 337.1	58.67 ± 1.45	9.8 ± 0.29	5.82 ± 0.49	4 ± 0.15	4.78 ± 0.22	140.3 ± 1.86	5.77 ± 1.40
	2B	325.5 ± 39.65	1370.8 ± 287.2	63.25 ± 3.35	9.88 ± 0.25	6.39 ± 0.39	3.98 ± 0.27	4.09 ± 0.17	137 ± 0.91	33.45 ± 8.11
	1	233.5 ± 8.61	325.1 ± 277	63.25 ± 2.87	9.575 ± 0.06	4.96 ± 0.28	3.73 ± 0.11	4.07 ± 0.17	137.5 ± 1.32	30.57 ± 6.42
	C	280 ± 32.62	2074.4 ± 333.4	60.33 ± 2.73	10.03 ± 0.19	5.55 ± 0.39	4.07 ± 0.07	4.25 ± 0.20	138 ± 1.16	17.76 ± 4.31

C=Control Tank; AST=aspartate aminotransferase; CK=creatine kinase; GLU=glucose; Ca^{2+}=total calcium; Ph=phosphorus; TP=total proteins; K^{+}=potassium; Na^{+}=sodium

Table 7.5 Ranges of selected hematological and blood biochemical parameters in the American alligator.

Values represent ranges for selected plasma analytes and PCV obtained from previous studies (Barnett et al. 1998; Dessauer; 1970; Guillette et al. 1997; Lance et al. 1983; Lance and Elsey 1999). Parameters are in bold, with units indicated in parentheses. We could not find a reference for creatine kinase (CK), therefore it was excluded from the table.

AST (U/L)	**GLU (mg/dL)**	**Ca^{2+} (mg/dL)**	**Ph (mg/dL)**	**TP (g/dL)**	**K^{+} (mmol/L)**	**Na^{+} (mmol/L)**	**PCV (%)**
≈140	60-70	8-12	3-9	5-30	3-5	≈141	≈18-20

AST=aspartate aminotransferase; CK=creatine kinase; GLU=glucose; Ca^{2+}=total calcium; Ph=phosphorus; TP=total proteins; K^{+}=potassium; Na^{+}=sodium

Figures

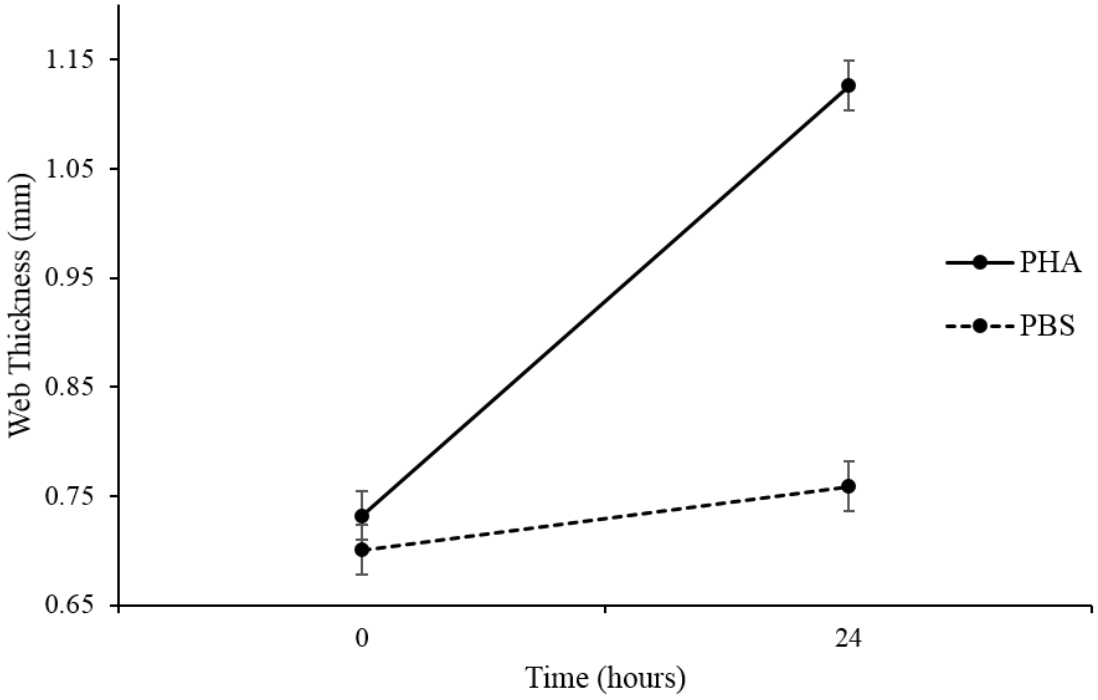

Figure 7.1 The effect of PHA and PBS on Swelling (Web Thickness in mm) prior to (0 hours) and 24 hours post injection.

Shaded circles are predicted means (± SE) of respective treatments. Swelling of PHA is indicated with a solid line, PBS with a dashed line.

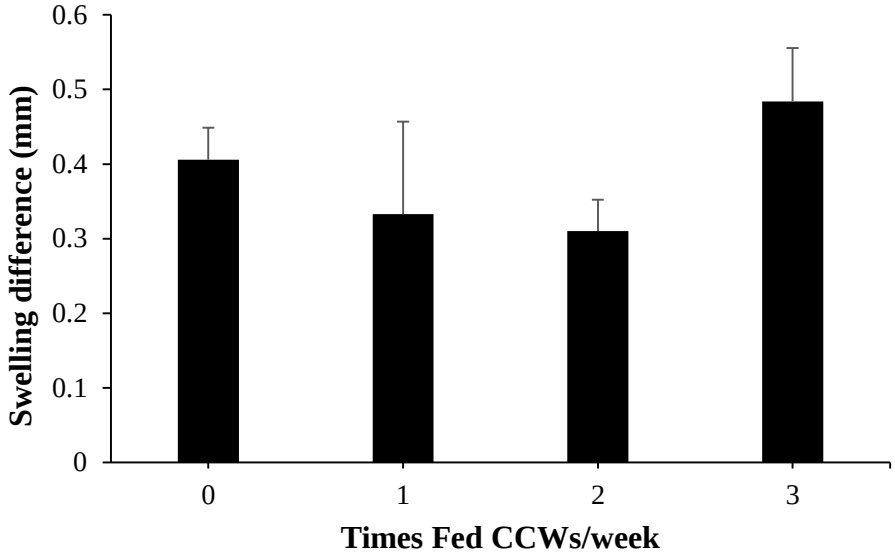

Figure 7.2 The effect of Feeding Treatment on the difference in Swelling 24 hours after injection.

Shaded bars represent predicted means (±SE) of Swelling difference (PHA minus PBS) 24 hours after initial injection. The x-axis indicates the different Feeding Treatments by the times each group was fed CCWs (coal combustion wastes) per week.

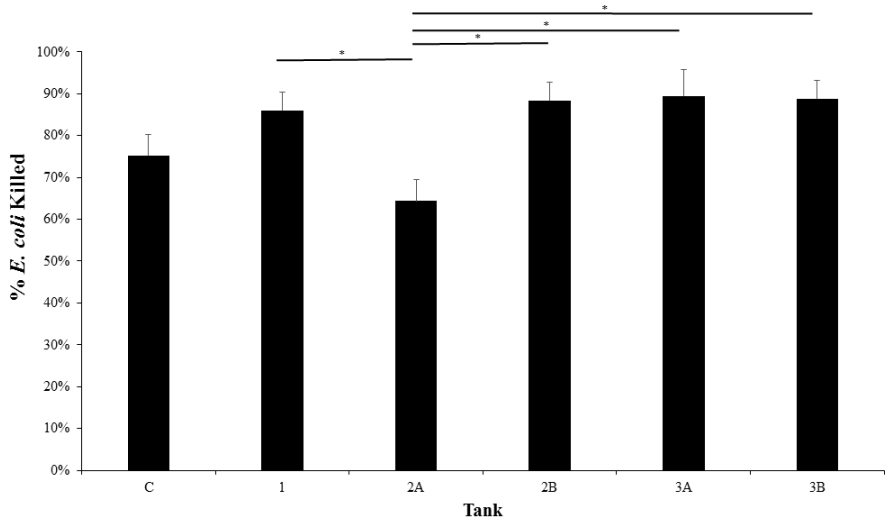

Figure 7.3 The effect of Tank on percent *E. coli* killed.

Shaded bars represent predicted means (±SE) of percent bacteria killed. Tanks are indicated on the x-axis and percent bacteria killed indicated on y-axis. Asterisks (*) and lines linking bars indicate significant differences between Feeding groups. “C” indicates the control tank (fed no CCWs/week).

CHAPTER 8

CONCLUSION

The ability of an organism to efficaciously mount an immune response following pathogenic exposure, immunocompetence, is of utmost importance in life history. However, multiple factors, such as anthropogenic or environmental stressors, may affect immune response. In this dissertation, I investigated how various types of stressors may affect the immune system of crocodilians. Crocodilians represent an ancient archosaurian lineage composed of at least 23 different species scattered globally in temperate and tropical climates. These long-lived, top trophic carnivores are important ecologically and economically. Furthermore, due to their long-lived and top trophic status, they may serve as important indicators of environmental quality.

Recent research, reviewed in this dissertation, has presented novel insight into the field of crocodilian immunology (Chapter 2). However much of this has been restricted to insights into innate immunity. In this dissertation we have validated (Chapter 4) and utilized immune techniques pervasive in toxicology, ecoimmunology, and in agricultural studies (Chapter 6 and 7). However, these techniques are not without their multiple confounders and caveats (Chapter 6). And whilst they provide some insight into crocodilian immunity, new and novel techniques, such as flow cytometry and other *in vitro* assays, must be developed to better understand the crocodilian immune system and the effect of stressors on that system (Chapter 6).

During the mid-20th century, the saltwater crocodile and American alligator underwent drastic population declines due to overhunting. However, alligators in the USA and saltwater crocodiles in Australia have rebounded quite remarkably. In fact, both are now important economic commodities that are sustainably harvested for meat and skin products (Chapter 6). In Australia, in particular, the crocodile industry is a relatively emergent industry, therefore many studies are necessary to understand how the farming environment may impact productivity (Chapter 6). As such, we validated a technique in Chapter 4 and utilized this technique, along with others, in Chapter 6 to determine the role the farming environment may have on baseline corticosterone, the predominant glucocorticoid in reptiles.

Whilst our results in Chapter 6 suggest that farmed hatlchings are not stressed, they have also highlighted a number of areas for future exploration. For example, although conditions were standardized throughout samplings, there were still stressors that were unable to be accounted for. Furthermore, it remains unkown as to what is actually contributing to the increased responsiveness to phytohemmaglutinin and enhanced bactericidal capabilities observed at each sequential sampling period. As our understanding of the crocodilian immune system is relatively rudimentary, further research examining distinct components of the immune system is necessary. The significant clutch effects on body size have been observed previously (see references within Chapter 6). However the observed clutch effects on corticosterone at Sampling 1 and those observed on bactericidal capabilities require further elucidation.

As crocodilians, such as the American alligator and saltwater crocodile, are long-lived and top trophic status organisms, various anthropogenic stressors are of concern

(Chapter 3 and Chapter 7). Futhermore, due to their economic status, along with rebounding population sizes, it is important to understand how anthropogenic impacts may affect crocodilians. Indeed, Guillette et al. have detailed multiple endocrine and reproductive toxicity studies in the alligators inhabiting Lake Apopka in Florida, USA, a lake that has received numerous pesticidal effluent from adjacent farm land and chemical spills (Chapter 3). A few previous studies and anecdotal observations of alligators inhabiting ash basins provided the impetus for our investigations into the effects of ingestion of prey contaminated with coal combustion wastes (CCWs) on the immune system and overall health (Chapter 7). Although alligators in the study accumulated significant amounts of trace elements present in CCWs over the course of the 2 years, we observed no effects of feeding treatment on the immune system or health of alligators.

The results observed in Chapter 7 suggest that exposure to CCWs may not detrimentally affect the health or immune status of alligators. However, it is important to emphasize that the long-lived nature of crocodilians and our short study time (relative to maximum age) may have affected any observed results. Futhermore, it is important to understand the contribution and effects of each individual trace element, as some elements accumulated over time, whereas others did not. As stated in Chapter 6 and 7, it is also important to implement novel techniques that are more sensitive or examine different parameters, such as gene expression, in determining if a particular contaminant has an effect on the immune system or health of an alligator. Furthermore, the observed tank effects highlight the variable inter-tank environments, even under standardized conditions. This, requires further examination to minimize variability, as this may influence observed outcomes and therefore any interpretations.

Future studies should expand the work described herein and continue to understand how these trace elements impact health.

An important factor affecting population size is microbial infection. Crocodilians are the closet extant relative to birds and birds are thought to be the main influenza virus reservoir (Chapter 5). Furthermore, waterfowl are a mainstay in the diet of many crocodilians. As many types of waterfowl are known to exhibit susceptibility to influenza infection, it is logical to posit that crocodilians, such as alligators, may be exposed to influenza. As reassortments of different influenza virus subtypes are a concern due to their pandemic potential, it is important to investigate novel species that may be susceptible to infection.

In Chapter 5, investigations were carried out to determine the susceptibility of American alligators to influenza virus infection. This was carried out by examining *in ovo* and *in vitro* routes of inection. Importantly, our results identify another potential host for influenza virus infection and subsequent replication. However, *in vivo* susceptibility to influenza virus in alligators remains unkown. Our results also highlight that alligators may be susceptible to both avian and human derived influenza strains. Therefore, could alligators serve as mixing vessels for reassortant viruses? Further research should examine this. We also know relatively nothing about the crocodile immune response following viral infection. However, anecdotal observations of conspecific infliction of wounds or limb removal and subsequent survival suggest a robust, yet understudied immune response. Therefore, future studies should aim to examine this.

In this dissertation we have investigated the role of various stressors and their effects on the immune system of the saltwater crocodile and the American alligator. However, we have also highlighted knowledge gaps and avenues for future exploration. Due to the status of crocodilians as valuable economic commodities and their role as important habitat modifiers, top trophic organisms, and environmental indicators, further examination of impact of various stressors on crocodilians is necessary.