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THE EFFECT OF CAPTURE, CONFINEMENT AND IMMOBILISATION ON ACUTE PHASE PROTEINS, AND IMMUNE AND HAEMOSTATIC RESPONSES IN THE IMPALA (*AEPYCEROS MELAMPUS*)

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Johannesburg, South Africa,

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CANDIDATES DECLARATION

I, Kelsey Lee Brown, declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of MSc (Med) Wildlife Stress Physiology at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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ABSTRACT

Background: Environmental and anthropogenic disturbances have a variety of direct and indirect impacts on wildlife. Various methods have been proposed to identify and quantify stressors that negatively impact wildlife.

Objectives: In this review, we examined the physiological stress response elicited during capture, confinement, and immobilisation on the acute phase response (APR), and immune and haemostatic responses in the impala.

Method: Blood- and faecal-centric approaches were used to determine: (1) the hypothalamic-pituitary-adrenal (HPA) axis activity by measuring cortisol in blood using a radioimmunoassay (RIA) and faecal glucocorticoid metabolites (FGM) using an RIA for corticosterone validated in many species, (2) the innate immune activity by measuring the circulating levels of leukocytes using manual white blood cell (WBC) counts and a haemocytometer, and leukocyte coping capacity (LCC) in response to challenges using reactive oxygen species (ROS) assay, (3) the secretion of acute phase proteins (APP) by measuring circulating levels of haptoglobin (Hp) and serum amyloid A (SAA) using an enzyme-linked immunosorbent assay (ELISA), and (4) the coagulation potential by measuring the intrinsic pathway using an activated partial thromboplastin time (aPTT) assay, the extrinsic pathway using a prothrombin time (PT) assay, fibrinogen conversion using a thrombin time (TT) assay, and fibrinogen concentration using a fibrinogen assay.

Results: In response to the chronic effects of confinement and the repeated stress of immobilisation we observed an initial increase in HPA activity and common coagulation pathway activity, and resultant depletion in body reserves. There was a decline in HPA activity as the trials progressed and corresponding immunesuppression. Thereafter, as animals habituated to stressors, we found that several of the parameters measured in the impala showed recovery to initial levels (including body weight and condition, FGM concentration, ROS generating capability of whole blood, and coagulability). In response to 30 min of immobilisation the impala showed decreased HPA activity and coagulability, and immune-enhancement.

Conclusion: These results demonstrate the feasibility of using physiological examinations to progress the understanding of short- and long-term impacts of anthropogenic disturbances on wildlife fitness, and improve animal welfare during conservation interventions.

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NOMENCLATURE

ACTH	adrenocorticotropin hormone
ANS	autonomic nervous system
APP	acute phase protein
APR	acute phase response
aPTT	activated partial thromboplastin time
BP	blood pressure
CNS	central nervous system
CRP	C-reactive protein
DIC	disseminated intravascular coagulation
ELISA	enzyme-linked immunosorbent assay
F	factor
FDP	fibrinogen degradation product
FGM	faecal glucocorticoid metabolites
GAS	general adaptation syndrome
GC	glucocorticoid
HDL	high-density lipoprotein
HPA	hypothalamic-pituitary-adrenal
Hp	haptoglobin
HR	heart rate
IL	interleukin

LCC	leukocyte coping capacity
MCC	Medicines Control Council
PMA	phorbol 12-myristate 13-acetate
PBS	phosphate buffer saline
PT	prothrombin time
RIA	radioimmunoassay
RLU	relative light units
ROS	reactive oxygen species
RR	respiratory rate
SAA	serum amyloid A
SAM	sympathetic-adrenomedullary
SNS	sympathetic nervous system
TF	tissue factor
TFPI	tissue factor pathway inhibitor
Th	helper T cell
TNF-α	tumor necrosis factor alpha
t-PA	tissue plasminogen activator
TT	thrombin time
vWF	von Willebrand factor
WBC	white blood cell

1 INTRODUCTION

As the number and diversity of animal species meriting conservation intervention increases, improved understanding of the impact of human-induced disturbance events on animal survival and reproduction (i.e. fitness) is essential (Dickens *et al.* 2010). Immobilisation, capture, relocation and confinement represent important components of conservation management (Teixeira *et al.* 2007). In terms of conservation, the capture and confinement of animals has various objectives, including: wound treatment from injuries, relocation, management of breeding stock within small enclosures, introduction of new genetics, sales of individual animals, veterinary emergencies, management work such as fitting radio collars or microchips, disease control, etc. (Moueix 2015). Physiological changes associated with the stress of capture/restraint have consistently been observed in studies conducted on non-domestic species (Dehnhard *et al.* 2001, Goymann *et al*. 1999, Terio *et al.* 1999, Turner *et al.* 2002). Despite widespread recognition that these and other anthropogenic disturbances are linked to circumstances due to the effects of physiological stress, understanding of the comprehensive impact on wildlife species is limited (Teixeira *et al.* 2007). Irrefutably, the wellbeing of individual animals during conservation interventions is well considered, but within published literature it is apparent that physiology is often underrepresented as a feature of direct concern (Chown & Gatson 2008, Cooke & O'Connor 2010, Tarszisz *et al.* 2014, Wikelski & Cooke 2006). A higher degree of consideration is essential to identify previously neglected or concealed conservation issues, and to better manage the impact of human-induced disturbance events on wildlife.

When applied to conservation management stress physiology, the measurement of stress metrics provides vital data on the causal mechanisms that underlie the current problems associated with intervention programs (Dantzer *et al.* 2014, Tarszisz *et al.* 2014). Current literature in the field of stress physiology reveals that while most studies have examined hormonal stress response activation, few have addressed the physiological consequences of adapting to these stressors (Teixeira *et al.* 2007). Few researchers have also had the opportunity to address the effects of short- (acute) and long-term (chronic) stress in wildlife, and consider both the adaptive and pathophysiological nature of elevated physiological "stress mediators," particularly the glucocorticoids (GC). An additional limiting factor of the application of stress physiology in conservation management is interspecies variation in the stress response. The physiological response to environmental conditions is both inter- and

intra- specific. Species-specific data in regard to the stress response is therefore required to overcome this limitation. Impala were specifically selected for this study to address the necessity of species-specific data, and to build on the existing knowledge of the physiological response to stress for the purpose of improving the welfare of these, and other antelope species, exposed to stressful events.

In this study we examined the physiological stress response elicited during capture, confinement and immobilisation on the acute phase response (APR), and immune and haemostatic responses in impala. We argue that a detailed understanding of physiological stress through measurements of stress indices plays a pivotal role in interpreting studies focused on stress and animal welfare by aiding in our understanding of the mechanistic consequences of anthropogenic disturbances. In order to evaluate immobilisation, capture, relocation and confinement in this context, blood- and faecal-centric approaches were used to measure stress metrics related to the APR, and immune and hemostatic parameters in animals which have experienced a capture event, as well as short- and long-term captivity and repeated immobilisation and handling. Our objective was to investigate the physiological stress response in impala during the course of both acute stress and chronic challenges.

1.1 Defining Physiological Stress

The concept of stress was originally proposed by Hans Selye (1936) in connection to his "general adaptation syndrome" (GAS), as the general term used to describe physiological accommodation to environmental demands. Elevated activity of the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system (ANS), in particular the sympathetic-adrenomedullary (SAM) axis, has most frequently been measured in studies conducted on stress and the stress response (Mormède *et al.* 2007, Teixeira *et al.* 2007). However, it is important to note that interpreting the activation of these systems is multifaceted (Mormède *et al.* 2007).

Stressors are perceived by an animal through vision, hearing, olfaction, and touch or pressure to the skin. These stimuli are assembled primarily in the hypothalamus and then project into the limbic system and neocortical level of the brain (Spraker 1993). Impulses then activate two arms of the acute stress response: the fast fight-or-flight response and the slower GC response.

The immediately-initiated fight-or-flight response is mediated by the activation of the SAM axis, which stimulates the adrenal medulla and results in the release of catecholamines (e.g. epinephrine and norepinephrine) (Dickens *et al.* 2010, Spraker 1993). Initiation of the fight-or-flight response allows for quick reaction to the stressor and includes elevation in heart rate (HR), vasoconstriction of vessels of the kidney, digestive system, connective tissues, and skin, and a corresponding vasodilation of vessels to the brain, skeletal muscle, heart, and lungs (Dickens *et al.* 2010, Spraker 1993). Catecholamines are catabolic and cause lipolysis and gluconeogenesis, facilitating the mobilisation of energy sources to the central nervous system (CNS) and somatic muscle. Epinephrine and norepinephrine also inhibit gastrointestinal motility and secretions (Dickens *et al.* 2010, Spraker 1993). The fight-or-flight response is then followed by the slower hormonal stress response, in which the adrenal gland secretes GC hormones as an end result of a hormonal cascade along the HPA axis (Dickens *et al.* 2010). The HPA axis begins with the stimulation of the hypothalamus, which signals the pituitary to secrete adrenocorticotropin hormone (ACTH). ACTH triggers the adrenal gland to release GC (i.e. cortisol) (Dickens *et al.* 2010, Seaward 2012, Spraker 1993). GC elevations increase the immediate availability of energy that can be used to resolve stressful situations by redirecting resources (Creel 2001). Negative feedback quickly suppresses GC release once the stressor diminishes or ceases (Dickens *et al.* 2010, Ganzel *et al.* 2010, Spraker 1993).

In spite of the widespread recognition of the activation of these systems in response to a stressor, a shortfall in scientific literature pertaining to stress lies in the current lack of a universally accepted definition of the term “stress” itself. Its overuse in popular culture has resulted in the term having connotations that makes it less useful in understanding how the body copes with psychosocial, environmental, and physical challenges (McEwen 2000, McEwen 2006). Many definitions are tautological. For instance, there is a tendency to consider any change in the HPA and SAM axes as a response to a stressful situation; however changes can also reflect their involvement in the homeostatic metabolic processes which are a natural part of life for all animals (Creel 2001, Mormède *et al.* 2007). Living organisms have regular patterns and routines that involve making the necessary preparations (i.e. the acquisition, utilisation, and storage of energy reserves) to carry out predictable life history stages such as breeding, migrating, moulting, and hibernating (McEwen & Wingfield 2003).

Predictability and control are important aspects to be considered when defining the physiological stress response.

The combined actions of the acute stress response make up the "Emergency Life-History Stage", whereby physiological and behavioural processes are functionally diverted throughout the body to focus on immediate survival needs, suspending otherwise normal life-history functions (e.g. reproductive physiology and immunological system maintenance) (Dickens *et al.* 2010, Martin 2009). In the short term, these responses are crucial for adaption, maintenance of homeostasis, and survival. However, the duration and intensity of the stimulus plays a pivotal role (Martin 2009). There is an inherent paradox whereby if a stressor persists or a series of acute stressors initiate multiple consecutive stress responses, an animal becomes chronically-stressed. Chronic stress has traditionally been thought to represent a dysregulation of the HPA axis (McEwen 1998, McEwen & Seeman 1999), primarily caused by a loss of negative feedback in which chronic stress decreases the number of GC receptors in key regulatory parts of the brain (e.g. the hippocampus and hypothalamus) (Romero 2004, Romero *et al.* 2009, Sapolsky *et al.* 1984). Chronically stressed individuals tend to experience a larger cumulative exposure to GC and, subsequently, harmful consequences, including immuno- and reproduction suppression and loss of muscle mass (Creel 2001, McEwen and Wingfield 2003, Sapolsky 2005, Sapolsky *et al*. 1986). The acute stress response therefore no longer aids in survival and instead can lead to detrimental physiological and behavioural consequences, potentially resulting in pathological conditions (Dickens *et al.* 2010, McEwen 2000, Teixeira *et al.* 2007). This subjective experience of stress, with physiological mediators being associated with both adaption and pathophysiology, further promotes the ambiguity of the term "stress". To better conceptualise acute versus chronic stress we will review the emerging concept of allostatic load, which was proposed as a means of augmenting the concept of "general adaptation syndrome" in response to stressors.

1.1.1 Allostasis and Allostatic Load

Allostasis, which literally means "maintaining stability through change" (McEwen 1998, pp. 171), has the potential to replace homeostasis as the core model of physiological regulation (Sterling 2004). The concept was originally introduced by Sterling & Eyer (1988) and refers to the active process of adaptation to acute stress, involving the output of stress hormones

which act to restore homeostasis (Korte *et al.* 2007, McEwen 2000, McEwen 2004, McEwen & Wingfield 2003). In response to either a predictable or unpredictable perturbation, an individual will enter an allostatic state, wherein they will have altered and sustained activity levels of primary mediators (i.e. catecholamines and GC), in an attempt to restore homeostasis within a range around a norm required for physiological functioning (McEwen 2004, McEwen & Wingfield 2003). Allostatic states can be sustained for limited periods if food intake and/or stored energy (i.e. fat) can fuel homeostatic mechanisms (McEwen 2004, McEwen & Wingfield 2003). Under most circumstances, the organism copes adequately with environmental stimuli by means of this coordinated plasticity, and continues along its life-history path. Allostatic load and allostatic overload refer to the cumulative result of an allostatic state (McEwen 2004, McEwen & Wingfield 2003). Within limits, routines which cause allostatic load involve adaptive responses to seasonal and other demands. However, if the additional load of unpredictable events in the environment (e.g. anthropogenic disturbances) is superimposed, then allostatic load can increase dramatically to become allostatic overload (McEwen 2004, McEwen & Wingfield 2003). Stress then becomes distress, whereby it incurs a biological cost so large that the animal needs to divert biological resources away from normal biological functions (e.g. the immune system) to cope with the stressor (Teixeira *et al.* 2007). Allostatic overload results in wear and tear on the regulatory systems in the brain and body, and predisposes the individual to various pathologies (McEwen 2004, McEwen & Wingfield 2003).

The concept of allostasis provides a more restricted and precise definition of the phenomena previously set out by the broad concept of stress. “Allostasis forms a continuum between events of the normal life cycle and unpredictable perturbations with clear transition points and outcomes” (McEwen & Wingfield 2003, pp. 12). Further, it provides a link between the essential protective and adaptive, and the damaging effects of physiological stress mediators (McEwen 2000, McEwen 2004, McEwen 2006, McEwen & Wingfield 2003).

1.2 Defining the Link between Stress, Capture and Confinement, and Immobilisation

Human influence on natural systems is increasing continuously. As a result, the capacity to understand what aspects of human influence cause stress in animals warrants increasing consideration (Wikelski & Cooke 2006). Where capture, confinement and immobilisation represent prominent emergency conversation interventions in wildlife, the challenge of stress

is centrally important to validating the feasibility of these practises. The capture of animals in the wild is normally a relatively rapid process, and one that is likely only to cause acute stress. Evidence from studies indicate that animals typically begin mounting a response via the HPA and SAM axes immediately upon capture and prior to direct human interaction, with elevated levels of catecholamines and GC, the primary stress mediators, consistently being seen (Dickens *et al.* 2010, Ganzel *et al.* 2010, Möstl & Palme 2002, Spraker 1993, Teixeira *et al.* 2007). In the short term, these acute stress responses serve the adaptive purpose of protecting an individual. However, the confinement of formerly free-ranging animals may cause chronic stress since it exposes individuals to both persistent stressors and consecutive acute stress responses. In confined animals, prolonged activation of the HPA and SAM axes no longer aids in survival and instead can increase an individual's risk of pathogenesis (Dickens *et al.* 2010, McEwen 2000, Teixeira *et al.* 2007). Information on stress responses to immobilisation is limited, however studies suggest that immobilisation has the potential to induce a substantial stress response and disturb normal regulatory systems, resulting in undesirable side effects (Kumar *et al.* 2002, Siegal-Willott *et al.* 2009, Taylor 1989).

1.3 Evaluating the Adaptive vs. Damaging Effects of Stress

As aforementioned, the nature of stress can be both protective in response to acute stressors and damaging in the long-term. The effects of this phenomenon have been observed throughout the body, however, for the purpose of this review we will be examining the effects of capture and confinement, and immobilisation on the APR, and immune and haemostatic responses (for those unfamiliar with the details of the APR, and immune system and haemostasis, we will provide a brief overview).

1.3.1 The Immune Response

The mammalian immune system functions to protect an animal's body from the invasion of antigens (e.g. pathogens) with layered defences of increasing specificity. Innate immunity provides an immediate, but non-specific response to an antigen. If the antigen successfully evades the innate response, vertebrates possess a second layer of protection, adaptive/acquired immunity, which is activated by the innate response and is antigen-specific. Both innate and adaptive immunity depend on the ability of the immune system to distinguish between self and non-self-molecules. The innate immune response involves

physical and chemical barriers, and it also includes cellular defences. It provides the first line of defence, which is not concerned with 'what' the antigen is, it merely prevents the antigen from entering body or destroys it before identifying it. Cells involved in innate immunity do not provide defence against any particular antigen, rather they function as all-purpose cells that can attack various different antigens and do so in a relatively short time frame when required (Segerstrom & Miller 2006). Phagocytic cells are the largest group of cells involved in innate immunity. If an antigen passes through the epithelial barrier, phagocytes such as neutrophils and macrophages ingest the antigens into vesicles and chemically destroy those (Abbas & Lichtman 2009). "The generalized response mounted by these cells is inflammation, in which neutrophils and macrophages congregate at the site of injury or infection, release toxic substances such as oxygen radicals that damage invaders, and phagocytose both invaders and damaged tissue" (Segerstrom & Miller 2006, pp. 3). In addition, these phagocytes and other cell types of the innate immune system, such as dendritic cells, secrete communication molecules called cytokines. Macrophages, in particular, secrete cytokines that stimulate inflammation and lymphocyte responses, in addition to other broad effects on the organism (Abbas & Lichtman 2009, Segerstrom & Miller 2006). These proinflammatory cytokines include interleukin(IL)-1, IL-6, and tumor necrosis factor alpha (TNF-α) (Segerstrom & Miller 2006). Further, these cells form an important link between innate and adaptive immunity: macrophages and dendritic cells act as antigen-presenting cells, which activate cells of the adaptive immune system (Abbas & Lichtman 2009). Other granulocytes of the innate immune system (e.g. mast cells, basophils and eosinophils) are important mediators of allergic responses (Minai-Fleminger & Levi-Schaffer 2009, Schroeder 2009).

Natural killer (NK) cells are another cell type involved in innate immunity. These NK cells detect stressed/infected cells or cells with DNA damage and lyse these cells by releasing toxic substances (Abbas & Lichtman 2009, Segerstrom & Miller 2006). Natural killer cells play a significant role in limiting the early phases of infection, before adaptive immunity becomes effective, and in removing self-cells which have become malignant (Segerstrom & Miller 2006).

Many circulating proteins bind antigens and help to eliminate them. Important among these are the proteins of the complement system, which bind to and are activated by antigens, and up-regulate phagocytosis and inflammation (Chaplin 2010). The complement system is also

activated by antibodies, and thus functions as part of adaptive immunity as well (Abbas & Lichtman 2009, Segerstrom & Miller 2006).

Adaptive immunity is characterised by antigen specificity and immunologic memory, however, the greater specificity of the response results in a slower onset than the innate immune system. Three types of lymphocytes mediate specific immunity: helper T (Th) cells, cytotoxic T cell (Tc) cells, and B cells (Segerstrom & Miller 2006). Lymphocyte receptors provide pathogen-specific recognition. Further, there are two types of adaptive immune responses: cell-mediated immunity, mediated by T lymphocytes, and humoral immunity, mediated by antibodies produced by B lymphocytes.

Tissue dendritic cells are activated by ingesting antigens at the site of infection. This induces their migration to local lymphoid tissues and their maturation into highly effective antigen-presenting cells (Janeway *et al.* 2001). Once in the lymphoid tissue, dendritic cells present, by means of a major histocompatibility complex (MHC) located on their surface, processed epitopes for cognate interactions with recirculating antigen-specific T cells (Bai *et al.* 2003, Steinman 2006). When activated, these naive T cell clones proliferate and differentiate to create a population of cells that can defend against a specific threat (Segerstrom & Miller 2006). Effector Tc cells divide to form numerous cloned Tc cells with the correct receptors. Some portion of these cells are active and will recognise body cells displaying epitopes of foreign antigen on their surface and induce apoptosis of those cells, while others are inactive memory cells that persist long term in the host to respond faster and more potently in case of re-exposure to that specific antigen (Abbas & Lichtman 2009, Obar & Lefrancois 2010). Effector Th cells are critical in coordinating and amplifying the activity of the immune response. Through the secretion of cytokines they stimulate the non-specific immune response to continue, and strengthen appropriate specific responses (Segerstrom & Miller 2006). Activated Th cells also help to recruit NK cells and phagocytes, and further activate Tc cells (Alberts *et al.* 2002, Obar & Lefrancois 2010). Further, when an antigen-presenting B cell interacts with an activated Th cell with the same specificity, the B cell is activated to differentiate into a plasma cell, proliferate and secrete large amounts of antibodies (Abbas & Lichtman 2009). Collectively immunoglobulin (Ig) -A, -E, -M, -G and -D function to: bind to free antigens to prevent their entry into cells, neutralise toxins associated with the relative infection, and opsonise (Segerstrom & Miller 2006).

The Acute Phase Response

Local inflammation is the first response of the immune system that an animal will mount upon exposure to trauma, infection, stress, immunological disorders and neoplasia (Ceciliani *et al.* 2012, Cray *et al.* 2009). When local defences are overwhelmed by noxious stimuli, the organism will respond by activating the APR, a complex systemic response intended at restoring tissue homeostasis and regulating the inflammatory response (Cray *et al.* 2009). Throughout evolution, the APR has been preserved as a core part of the innate immune response and is observed across all animal species (Petersen *et al.* 2004).

In response to an inflammatory stimulus, activated immune cells (particularly monocytes) generate cytokines (Petersen *et al.* 2004). This non-specific immune response involves various organs, distant from the site of inflammation, and is accompanied by a host of behavioural, physiological, biochemical and nutritional changes (Ceciliani *et al*. 2012). The most evident phenomena include pyrexia, leukocytosis, and the quantitative and qualitative modification of APP (Ceciliani *et al.* 2012, Ceciliani *et al.* 2002, Dantzer *et al.* 2008, Martin *et al.* 2011, Murata *et al.* 2004). Pro-inflammatory cytokines act as messengers, stimulating hepatocytes and peripheral tissues to synthesize acute phase proteins (APP), subsequently activating the APR (Bertelsen *et al.* 2009, Krogh *et al.* 2013, Piñeiro *et al.* 2007, Petersen *et al.* 2004). During the APR, the serum concentration of the APP changes significantly. In particular, the APP produced by hepatocytes, which are present at substantially different concentrations in the blood. They can be classified according to the magnitude of their increase (positive APP; e.g. haptoglobin (Hp), serum amyloid A (SAA), and C-reactive protein (CRP)) or decrease (negative APP; e.g. albumin) in serum concentration during an APR (Ceciliani *et al.* 2012, Petersen *et al.* 2004, Piñeiro *et al.* 2007). Acute phase proteins are thought to play a significant role in several aspects of the systemic inflammatory response, including the opsonization of several pathogens, the detection of potentially toxic substances and the overall regulation of different stages of inflammation (Ceciliani *et al.* 2012).

SAA is a major APP produced in large quantity predominantly by hepatocytes during the APR to inflammatory stimulus (Sato *et al.* 2016). Although information is limited on the precise role that SAA plays during the course of inflammation, it has been suggested that it may have a homeostatic role (Ye & Sun 2015). One specific mechanism in which it functions to maintain homeostasis is through its association with high-density lipoprotein (HDL),

whereby it enhances the antioxidant potential of these molecules (Sato *et al.* 2016). Hepatic production is also responsible for the synthesis of Hp as a part of the APR (Cray *et al.* 2009). The primary function of Hp is to prevent the loss of iron by binding with free haemoglobin in the blood to form exceptionally stable complexes (Laurel & Nymann 1957). Haptoglobin is thus believed to have a bacteriostatic effect by restricting the availability of iron necessary for bacterial growth (Bullen 1981, Petersen *et al.* 2004).

Capture and Confinement

"Conceptualizations of the nature of the relationship between stress and the immune system have changed over time." (Segerstrom & Miller 2006, pp. 5). Early studies supported the initial model in which stress was interpreted as being broadly immunosupressive (Borysenko & Borysenko 1982, Cohen *et al.*1991, Fox 1981, Khansari *et al.* 1990, Kort 1994, Maier *et al.* 1994). The heightened susceptibility of chronically stressed individuals to infectious and neoplastic diseases was assumed to be as a result of diminished immune responses (Andersen *et al.* 1994, Cohen & Williamson 1991). The first models for how stress and immunity are related were substantiated by various hypotheses, including: that immunity is diminished during stress responses to redirect resources to processes that are more critical to survival, further that immune suppression may represent the catabolization of immune cells and tissues to provide protein and glucose for more immediately valuable activities (Sapolsky *et al.* 2000), and the minimisation of autoimmune damage (Martin 2009). Despite the established and continued support for the global immunosuppression model, it has recently been challenged by the concept that relations between stress and the immune system should be adaptive, particularly within the context of fight-or-flight stressors, and the more recent interest in the balance between humoral and cellular immunity (Segerstrom & Miller 2006). A broad decline in immune function that the initial model predicts in response to all stressors is not an evolutionary adaptive characteristic in life-threatening circumstances (Dhabhar & McEwen 1997). Down-regulation of the immune system to provide resources for more valuable processes does not occur rapidly enough for this to be a viable explanation (Råberg *et al.* 1998). Some of the proposed mechanisms for immunosuppression (e.g. apoptosis of leukocytes) also expend rather than conserve energy (Sapolsky *et al.* 2000). Further, it makes little sense that organisms would suppress a system that could be integral to recovery from stressors (Dhabhar & McEwen 1997, Wingfield *et al.* 1998). Consequently, Dhabhar and McEwen (1997, 2001) proposed a more refined interpretation of stress-immune interactions:

a biphasic model in which acute stress enhances and chronic stress suppresses the immune response (Martin 2009, Segerstrom & Miller 2006). In a series of experiments with mice, they found that in response to acute or short-term stress neuroendocrine-immune pathways mediate immuno-enhancement. This includes; the redeployment of leukocytes from the blood to organs (e.g. skin and lymph nodes) where they are needed to fight an infection or other challenge, increases in innate immunity, pathogen presentation, effector T cell function, antibody production, and cytokine production (Dhabhar & McEwen 1997, Martin 2009, Segerstrom & Miller 2006). Conversely, in response to chronic or long-term stress, immunosuppression may be mediated by mechanisms involving inhibition of T cell activation, pathogen presentation, activation of lipocortins, induction of lymphocyte apoptosis, or suppression of effector T cell function (Dhabhar & McEwen 1997, Martin 2009, Segerstrom & Miller 2006).

An addendum to the biphasic model posits that acute stress enhances parameters of innate immunity, while diminishing adaptive immunity (Segerstrom & Miller 2006). This is based on innate immune responses being better able to manage the potential consequences of life-threatening situations than adaptive immune responses. Innate immunity requires only minimal time and less energy to be diverted from other bodily systems that support the fight-or-flight response, and are subject to fewer inhibitory constraints. Conversely, adaptive immunity is time and energy demanding, proliferation in particular (Segerstrom & Miller 2006).

Further, stress hormones (particularly GC) are integral to immune regulation (Sternberg 2006), although interactions are complex and dynamic (Martin 2009). During periods of stress, increased circulating levels of GC are known to occur due to the activation of the HPA axis (Dhabhar & McEwen 1997, Shanks *et al.* 1994). Circulating pro-inflammatory cytokines further instigate the release of GC (Glaser & Kiecolt-Glaser 2005). During an acute response, circulating GC levels typically rise several fold, but return to basal levels hours after the stressor is removed. However, in the case of a stressor persisting for days or weeks, individuals can exhibit elevated baseline GC, a stronger acute increase in plasma GC following an environmental challenge and a reduced ability to terminate the stress response (Dickens and Romero 2013, Sapolsky 2002, Sapolsky *et al*. 1986). Many in the scientific community speculate that chronic levels of high stress disrupt the delicate feedback balance, resulting in the failure of feedback inhibition to operate, and the continued release of cortisol

(Creel 2001, Sapolsky *et al.* 1986). These adrenal hormones have inhibitory effects on a broad range of immune responses (Acevedo-Whitehouse & Duffus 2009). The immunosuppressive effects of GC include the regulation of lymphocyte maturation, selection and proliferation (Griffin 1989). Further, by way of an inhibitory feedback mechanism, GC foster the down regulation of lymphocyte function, particularly of pro-inflammatory and cellular responses (Elenkov 2004, Martin *et al.* 2011, Sternberg 2006). Inflammation is particularly sensitive to cortisol (Glaser & Kiecolt-Glaser 2005), and GC actions have traditionally been regarded as anti-inflammatory. Glucocorticoids suppress inflammation by multiple mechanisms that impact both the innate and adaptive immune responses (Busillo & Cidlowski 2013). This includes inhibitory action on the production of various cytokines (e.g. IL-1, IL-6, and TNF-α) (Elenkov 2004, Martin *et al.* 2011, Sternberg 2006), and histamine secretion thereby regulating the activation of inflammatory cells (Griffin 1989). However, emerging evidence suggests that GC also can exert pro-inflammatory effects in response to stress (Sorrells & Sapolsky 2007). Glucocorticoids are known to stimulate the hepatic production of APP, including SAA, independent of the actions of pro-inflammatory cytokines (Jain *et al.* 2011). It has been consistently observed that in conjunction with the dysregulation of the GC hormone, often accompanying captivity, a pro-inflammatory state exists in the animals due to the impaired coordination of the attenuation and resolution of the inflammatory response (Martin *et al.* 2011). Recently, the potential application of APP has been suggested for diagnosis, prognosis, and assessment of animal health (Cray *et al*. 2009). Several studies have already demonstrated the utility of major APP quantitation in monitoring neoplasia and stress, with regard to animal welfare (Bernal *et al.* 2011, Eckersall *et al.* 2000, Murata *et al.* 2004, Piñeiro *et al.* 2007, Stanton *et al.* 2013). Piñeiro *et al.* (2007) conducted a study aimed at characterizing the response in swine following prolonged transport by road under commercial conditions. Elevated serum APP concentrations were observed in two groups of boars immediately after arrival at a destination farm compared with within-animal control samples obtained one month later. The first group of pigs were transported under average conditions (Transport 1, 24 hrs), while the second group was transported for a longer time period (Transport 2, 48 hrs) but in superior transport conditions. The effects on serum APP concentrations were more pronounced in the first group. From these result Piñeiro *et al.* concluded that, "Independent of the cause of the APP response that follows transport, whether due to tissue damage or not, it is clear that the increase in APP levels reflect compromised animal welfare" (2007, p 673).

Various mechanisms have been put forward to interpret how chronic stress is associated with the body losing its ability to regulate the inflammatory response. Cohen *et al.* (2012) proposed a model wherein prolonged psychological stress results in glucocorticoid receptor resistance (GCR) that, subsequently, results in the failure to down-regulate the inflammatory response. Specifically, immune cells have decreased tissue sensitivity to cortisol. Without sufficient GC regulation, the duration and/or intensity of the inflammatory response increases (Cohen *et al.* 2012). Thus, although chronically stressed individuals experience a higher cumulative exposure to GC hormones, Cohen *et al*. (2012) suggest that it is not the level of the hormones which matter, but rather how the target tissue responds to cortisol.

From a physiological perspective, a causal relationship has been proposed between excessive adipose tissue, and the pro-inflammatory state and overproduction of cytokines observed in captive animals (Schook *et al.* 2015). In response to an acute stressor, GC stimulate the rapid immobilisation of energy reserves, to allow the body to adaptively cope with adverse conditions (McEwen 2000, McEwen & Wingfield 2003). Blood glucose is elevated by increased lipid and protein catabolism, and hepatic gluconeogenesis (Arnemo & Caulkett 2007, Spraker 1993). Several reviews have suggested a correlative relationship between inflammation and insulin resistance (Rajala & Scherer 2003, Ramos *et al.* 2003, Xu *et al.* 2003). Inflammatory cytokines play an essential regulatory role in insulin/glucose homeostasis by inhibiting insulin signalling and cellular uptake of glucose, resulting in further hyperglycemia and compensatory hyperinsulinemia (Hotamisligil *et al.* 1996, Lang *et al.* 1992, Spraker 1993). Concomitantly, high glucose has been seen to have a stimulatory effect on the transcription factor NF-κB in hepatocytes, resulting in the expression of pro-inflammatory APP (e.g. CRP, SAA), further up-regulating the inflammatory response (Iwasaki *et al*. 2007). Furthermore, GC increases appetite for food and food seeking behaviour by action on the brain (McEwen 2000, McEwen & Wingfield 2003). While stress hyperglycaemia is an essential survival response, chronically elevated cortisol and insulin leads to the storage of energy as fat in the adipose tissue, with the net effect of increased body fat deposition at the expense of tissue proteins (McEwen 2000, McEwen & Wingfield 2003). Schook *et al.* (2015) conducted a review on black rhinoceros (*Diceros bicornis*) living in zoos, wherein they proposed that the captive environment contributes to increased inflammation and decreased insulin sensitivity, and an associated increase in morbidity and mortality in the species.

Also worth consideration, is the increasing evidence that points to an extensive cross-talk between inflammation and coagulation (Engelmann & Massberg 2013, Levi *et al*. 2004). "Activation of coagulation and fibrin deposition as a consequence of inflammation is well known and can be viewed as an essential part of the host defence of the body against, for example, infectious agents or non-identical cells, in an effort to contain the invading entity and the consequent inflammatory response to a limited area" (Levi *et al*. 2004, pp. 2698). However, recent evidence suggests that not only does inflammation lead to activation of coagulation, but coagulation also has a considerable effect on inflammatory activity. There have been many *in vitro* observations of coagulation proteases not only interacting with coagulation protein zymogens but also with specific cell receptors to induce signalling pathways and result in the up-regulated expression of pro-inflammatory mediators (Jones & Geczy 1990, van der Poll *et al.* 2001). Further, there is mounting evidence that the protein C pathway is functionally important in the modulation of the inflammatory response (Esmon 2002). Activated platelets also play an essential role in inflammation, particularly in chronic inflammation (Wagner & Burger 2003). Platelets contain and are capable of releasing high concentrations of CD40 ligand, a pro-inflammatory mediator. These mediators then increases inflammatory cytokines, such as IL-6 and IL-8 (André *et al.* 2002, Henn *et al*. 1998), and induces tissue factor (TF) synthesis (Miller *et al*. 1998, Pendurthi *et al*. 1997). Fibrinogen and fibrin have been observed to directly stimulate the expression of pro-inflammatory cytokines (e.g. TNF-α and IL-β) and induce production of chemokines (e.g. IL-8 and MCP-1) (Szaba & Smiley 2002).

1.3.2 The Haemostatic Response

In mammals, an advanced haemostatic surveillance system has evolved that protects animals from excessive blood loss and infection by almost instantaneously sealing damaged vessel walls (Engelmann & Massberg 2013, Gentry 2004, Hussein *et al.* 2010, Lukas 2010). The coagulation pathway, which mediates haemostasis, generates a clot that prevents blood loss following vascular injury and eventually restores endothelial integrity in the host (Engelmann & Massberg 2013, Gentry 2004, Hussein *et al.* 2010, Lukas 2010). "Haemostasis is a fundamental defence mechanism of all vertebrates and involves two complementary processes: the formation of a blood clot, or thrombus, to stem blood loss from a damaged vessel and the process of thrombus dissolution, or fibrinolysis, once endothelial repair has occurred" (Gentry 2004, pp. 238). The basic mechanism is similar in all species, and involves

both activated cells and plasma proteins that participate in highly complex and interdependent events localized to the site of trauma (Gentry 2004, Hussein *et al.* 2010, Smith 2009), culminating in the conversion of a soluble protein into an insoluble polymer (Iwanaga 1993, Theopold *et al.* 2002).

Haemostasis is maintained in the body via the following key mechanisms: (1) initial vasoconstriction, (2) platelet activation plug formation, (3) assembly and activation of the coagulation cascade factors, (4) fibrin clot formation at the site of injury, and (5) dissolution of the clot and vascular repair (Gentry *et al.* 1997). For the purpose of this review we will be discussing a simplified model of the haemostatic mechanism. Numerous reviews are available that describe the haemostatic process in more detail (Crawley *et al.* 2007, Gentry *et al.* 1997, Hoffman & Monroe 2007, Hopper & Bateman 2005, Seré & Hackeng 2003).

The endothelium not only functions as a selectively permeable barrier between blood and underlying tissue, but also plays a crucial physiologic role in maintaining homeostasis of circulation (Gentry *et al.* 1997). The endothelial cells of intact vessels regulate blood flow by providing an antithrombotic surface, which inhibits platelet aggregation and the coagulation cascade, through the expression of heparan sulphate, prostacyclin, nitric oxide, thrombomodulin and tissue plasminogen activator (t-PA) (Ando & Kamiya 1993, Cines *et al.* 1998, Colvin 2004, Gentry *et al.* 1997). Perturbations, such as those that occur at sites of vessel damage, disrupt these activities and induce endothelial cells to generate a prothrombotic and antifibrinolytic microenvironment (Ando & Kamiya 1993, Cines *et al.* 1998, Colvin 2004, Gentry *et al.* 1997). Vasoconstriction, produced by the contraction of vascular smooth muscle cells, is the initial response of blood vessels to injury. Vascular spasm is a brief autonomic neurogenic reflex, initiated by local receptors of the sympathetic nervous system (SNS) and the action of catecholamines that temporarily reduces the amount of blood flow through the area of trauma, helping to limit the amount of blood loss (Cines *et al.* 1998, Gentry *et al.* 1997, Lukas 2010). Endothelial cells further function in haemostasis by synthesizing and activating agents and expressing receptors (e.g. von Willebrand factor [vWF], collagen and TF), enhancing coagulation (Lukas 2010). The endothelium also facilitates leukocyte extravasation, whereby circulating leukocytes (particularly monocytes and neutrophils) are rapidly recruited to the site of trauma and bind by means of specific adhesion molecules expressed on the surface of the endothelial cells (Engelmann & Massberg 2013, van Hinsbergh 2012). Monocytes and neutrophils are subsequently incorporated into

growing intravascular clots during thrombus development (Engelmann & Massberg 2013). In parallel with these processes, the endothelium participates in fibrinolysis by releasing anticoagulant agents (e.g. t-PA) (Lukas 2010).

Primary haemostasis involves the process of platelet activation, adhesion, and aggregation. In mammals platelets are small, disc-shaped anucleate cells that circulate in the blood and function as sentinels of vascular integrity (Engelmann & Massberg 2013). When vascular injury occurs, inactive platelets circulating in the blood are recruited to the damaged site and adhere to endothelial connective tissue by interacting with collagen, vWF and platelet glycoprotein binding sites (Colvin 2004, Engelmann & Massberg 2013). The subsequent aggregation of platelets forms a fragile plug to help stem haemorrhage from the vessel (Colvin 2004, Lukas 2010). Adherent platelets are activated by collagen, and release their granule contents, including adenosine diphosphate (ADP), thromboxane A_2, and serotonin (Colvin 2004, Engelmann & Massberg 2013, Gentry *et al.* 1997, Lukas 2010). Substances released from platelets mediate continued platelet activation and aggregation, and further enhance vasoconstriction of the blood vessels (Lukas 2010).

“Whereas platelet aggregation provides a provisional closure of the wound, coagulation ensures that it remains mechanically stable, sealing the defect using the glue-like substance fibrin, which is the major end product of the coagulation cascade” (Engelmann & Massberg 2013, pp. 35). Historically, the two arms of secondary haemostasis have been described in literature as the intrinsic and extrinsic coagulation pathways. As the role of the various proteins involved in the coagulation cascade has been elucidated, the classical model of thrombin generation and fibrin formation has been revised. More recently these pathways are referred to as the contact activation (intrinsic) and TF (extrinsic) pathways (Gentry 2004, Gentry *et al.* 1997). As indicated in Figure 1.1, the pathways differ in the mechanisms by which they are initiated and following subprocesses but converge to form a common pathway at the point of factor (F) X activation (Lukas 2010, von Känel *et al.* 2001a). FXa and co-factor FVa form the prothrombinase complex, which activates prothrombin (FII) to thrombin (FIIa) (Engelmann & Massberg 2013, Gentry 2004, Gentry *et al.* 1997, Levi *et al.* 2004). Thrombin’s most significant physiological function is the conversion of soluble fibrinogen (FI), via soluble fibrin monomers, into the insoluble fibrin (Engelmann & Massberg 2013, Epstein & Brainard 2011, Gentry 2004, Levi *et al.* 2004, von Känel *et al.* 2001a). Fibrin is

subsequently stabilised by cross-linking with FXIIIa, to form a blood clot or thrombus (Epstein & Brainard 2011, von Känel *et al.* 2001a).

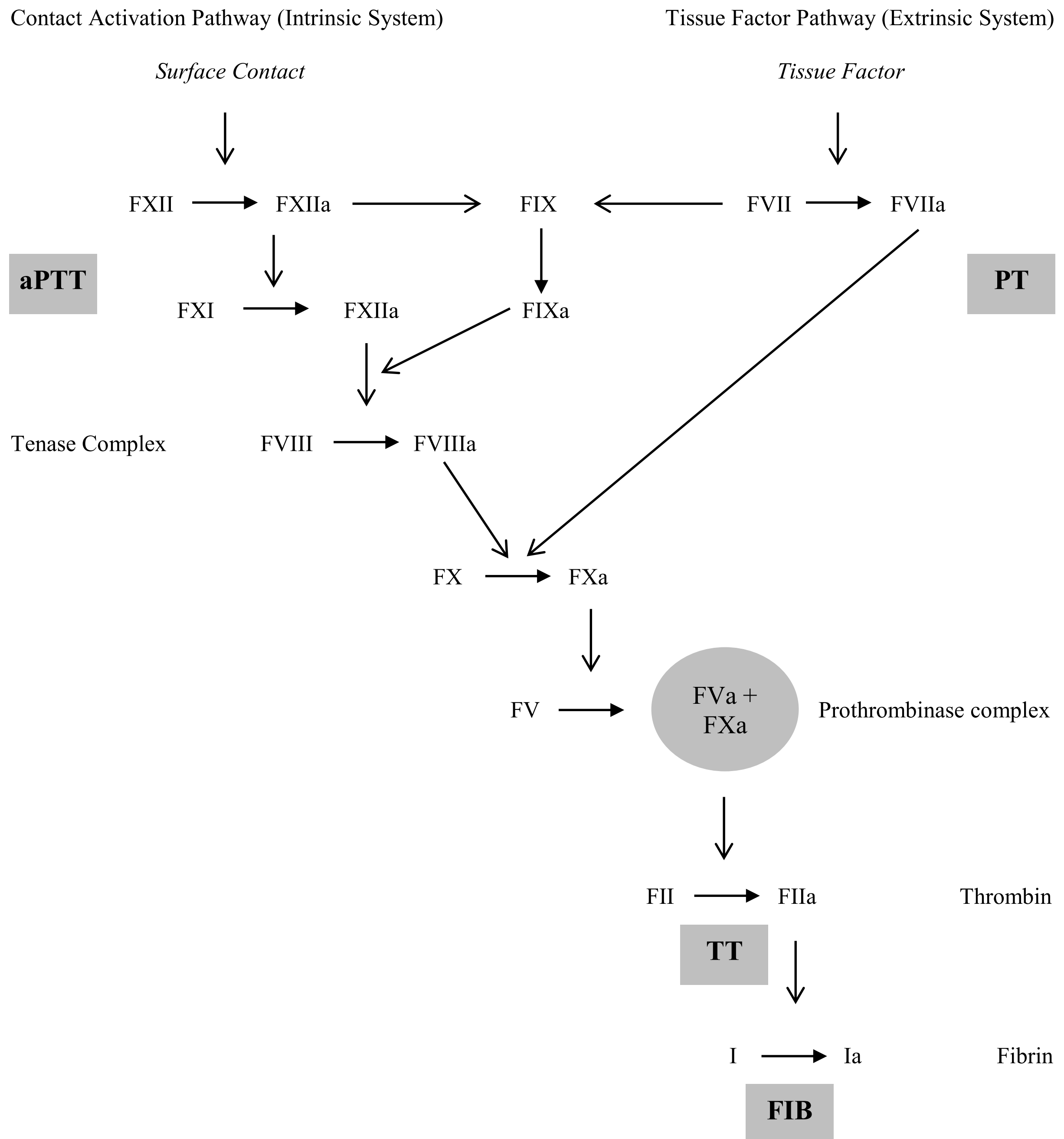


Figure 1.1 Schematic diagram of the coagulation cascade. The initiation of blood clot formation occurs following vascular injury and the exposure of TF to circulating blood. Activation of both intrinsic and extrinsic pathways leads to the fibrin clot formation in the common pathway. The majority of proteins are designated as factors (F) and Roman numerals. [aPTT: activated partial thromboplastin time, PT: prothrombin time, TT: thrombin time, FIB: fibrinogen concentration]. (Adapted from Gentry 2004, pp. 239).

Thrombin also initiates several positive feedback reactions to sustain its own formation and facilitate the rapid growth of thrombus (Dahlbäck 2005, Engelmann & Massberg 2013, Gentry 2004, Gentry *et al.* 1997, Levi *et al.* 2004). A dynamic equilibrium exists between the sequential and co-ordinated interactions of the procoagulant proteins that initiate and amplify thrombin generation, and a group of circulating anticoagulant proteins (e.g. tissue factor pathway inhibitor [TFPI], vitamin K-dependent protein C and antithrombin) (Engelmann & Massberg 2013, Esmon 2005, Gentry 2004). These inhibitory proteins function to ensure that thrombin formation is confined to the site of vascular trauma, thereby preventing uncontrolled or disseminated coagulation which can prove life-threatening (Engelmann & Massberg 2013, Esmon 2005, Gentry 2004, Gentry & Downie 1993).

Capture and Confinement

Extensive research conducted on the haemostatic response suggests that short-term arousal activities (e.g. acute stressors, acute exercise) concurrently activate coagulation and fibrinolysis within a physiological range, resulting in net hypercoagulability (Austin & Patterson 2013, Lukas 2010, Thrall *et al.* 2007, von Känel *et al*. 2001a, Womack *et al.* 2003). Through a series of experiments conducted in the first half of the 20th century, Walter B. Cannon was the first to effectively demonstrate that stimulation of the splanchnic nerve, pain, fear and enragement all shortened blood clotting times (Cannon 1953). Cannon's evolutionary interpretation of these observations was that stress-hypercoagulability protects a healthy organism from excess bleeding should injury occur in fight-or-flight situations (Cannon & Mendenhall 1914, Lukas 2010, von Känel *et al*. 2001a). A number of naturalistic studies to assess the short-term effects of mental arousal on coagulation activity have been conducted throughout the 20th century (Dreyfuss 1956, Kast & Zweibel 1954, Latner 1947, MacFarlane & Biggs 1946, Macht 1952, Ogston *et al.* 1962, Truelove 1951, Urano *et al.* 1990). Despite the use of insensitive markers and quality concerns in early studies, the concomitant activation of coagulation and fibrinolysis by mental arousal in naturalistic studies is in keeping with recent findings in standardized laboratory stress protocols (Davis 1999, Jern *et al.* 1989, Jern *et al.* 1991, Larsson *et al.* 1990, Muldoon *et al.* 1995, Musumeci *et al.* 1987, Palermo *et al.* 1989, Quartarone *et al.* 1978).

Regarding individual haemostasis molecules, increased activity has consistently been observed for procoagulant factors XIII (Jern *et al.* 1989, Steptoe *et al.* 2003) and VIII (Jern *et al.* 1989), fibrinogen (Davis 1999, Jern *et al.* 1989, Steptoe *et al.* 2003), vWF (Jern *et al.* 1989, Musumeci *et al.* 1987, Steptoe *et al.* 2003), and platelets (Brydon *et al.* 2006, Camacho & Dimsdale 2000). In addition, acute mental stress also elicits an increase in profibrinolytic factors, including t-PA (Jern *et al.* 1989, Jern *et al.* 1991, Palermo *et al.* 1989, Womack *et al.* 2003). The more sensitive markers of coagulation activation, fibrin D-dimer and TAT, were also elevated in plasma after acute mental stress (von Känel *et al.* 2003, von Känel *et al.* 2001b). The observed increase in D-dimer in particular supports the conclusion that, despite the concurrent activation of coagulation and fibrinolysis in response to acute stress, procoagulant mechanisms exceed anticoagulant forces (von Känel *et al.* 2001a). Healthy individuals that experienced acute mental stress also demonstrated vasoconstriction or alleviated vasodilation (Ghiadoni *et al*. 2000, Gottdiener *et al*. 2003, Sarabi & Lind 2001).

From a review of the literature on this topic, it can be observed that a relationship exists between acute stress-induced activation of the SAM axis and a hypercoagulable state (Austin & Patterson 2013, Lukas 2010, von Känel 2015, von Känel *et al*. 2001a). Positive associations between stress-induced catecholamine spill-over, accelerated blood clotting time, and stimulated fibrinolysis have been reported in a variety of laboratory and real-life situations (Larsson *et al.* 1990, Sidorenko & Revenko 1992, von Känel *et al.* 2001a). Elevated plasma epinephrine levels dose-dependently stimulate vascular endothelial β(2)-adrenergic receptors, subsequently triggering vascular spasm and the release of procoagulant FVIII, haemostatically active vWF and profibrinolytic t-PA into circulation (von Känel & Dimsdale 2000). Additionally, catecholamines stimulate hepatic release of FVIII and affect hepatic clearance of t-PA, and likely D-dimer (Austin *et al.* 2013). Circulating t-PA is further increased in response to a stressor by sympathetic nerves in artery walls (Hao *et al.* 2005). Catecholamines also activate platelets by stimulating α(2)-adrenergic receptors (von Känel & Dimsdale 2000).

Acute stress-induced hypercoagulation is further facilitated by the effects of haemoconcentration. In a healthy individual, the increase in blood pressure (BP) accompanying acute stress results in the net efflux of intravascular fluid through capillary pores into interstitial spaces, with a resulting increased concentration of non-diffusible large

(i.e. 69 kDa) haemostatic molecules (Allen & Patterson 1995, Austin & Patterson 2013, Austin *et al.* 2013).

It has been proposed that in healthy subjects, stress-induced procoagulant responses showed an inability to adapt or habituate to repeated stressors of the same type (Hamer *et al.* 2006, Lukas 2010, von Känel 2015, von Känel *et al.* 2004). Von Känel *et al.* conducted an investigation based on this premise, through which they confirmed that; "the magnitude of stress-induced changes and subsequent recovery showed no habituation in any coagulation variable" (2004, pp. 1333). An evolutionary paradigm has been posited to support this observation, in which a hypercoagulable state during and after stress protected our ancestors from exaggerated bleeding when injured as a part of the fight-or-flight response (Preckel & von Känel 2004, von Känel 2015, von Känel *et al.* 2001a, von Känel *et al.* 2004). Therefore, habituation of the coagulation response across stress repetitions would confer a disadvantage rather than a benefit with regard to survival of the human species (von Känel *et al*. 2004). Although normal haemostasis physiology is an important biological process mediated by elevated coagulation and fibrinolytic potentials that exist in a balanced equilibrium to prevent bleeding after vessel injury (Dahlbäck 2005, Gaffney *et al.* 1999), haemostatic variables and the prothrombotic tendency they bring about can also be associated with thrombosis. Thrombosis is considered to be a pathological deviation of haemostasis and is characterised by intravascular thrombus (clot) formation and vessel occlusion (Engelmann & Massberg 2013). Various investigations have shown that the effect of acute stress on haemostatic parameters is modulated by other risk factors, such as chronic stress (Lukas 2010, von Känel *et al*. 2001a). Chronic stressors are related to an increase in procoagulant factor VII, D-dimer, and VWF; and impaired fibrinolytic potential (indicated by increased plasminogen activator inhibitor-1 [PAI-1] and t-PA) (Lukas 2010, von Känel *et al*. 2001a). "Thus, the balance between coagulation and fibrinolytic activity consequently changes with chronic stress and promotes a hypercoagulable state which, moreover, exceeds the physiologic net hypercoaguability in healthy individuals observed during acute stress" (Lukas 2010, pp. 60). The exaggerated hypercoagulability because of dampened fibrinolytic activity associated with chronic stress (Austin *et al*. 2013, von Känel *et al.* 2003, von Känel *et al*. 2001a), has been observed to result in aberrant coagulation.

As aforementioned, there is ample evidence that coagulation and inflammation are intricately related processes that may considerably affect each other (Borissoff *et al.* 2011, Engelmann

& Massberg 2013, Levi *et al.* 2004, von Känel 2015). Inflammatory mediators have been shown to elevate platelet count and reactivity, down-regulate anticoagulant mechanisms, initiate the coagulation system, facilitate propagation of the coagulant response and impair fibrinolysis (Esmon 2005, Levi *et al.* 2004). Probably the most notable contribution of inflammation to thrombin generation is the induction of TF expression on the cell surface of leucocytes, in particular monocytes (Lindmark *et al*. 2000, Parry & Mackman 1998). If negatively charged PLs are exposed on activated monocytes, TF contributes significantly to a procoagulant state (Esmon 2005). Another significant contribution of inflammation is elevated fibrinogen concentrations. In addition to the role of fibrinogen as an APP in the inflammatory response, it also functions as a pivotal precursor in the coagulation cascade. Increased fibrinogen concentrations are consistently observed in response to inflammation (Epstein & Brainard 2011, Factor 2007, Hantgan *et al*. 2001), which have, subsequently, been associated with an increased risk of thrombus (Esmon 2005). Elevated plasma concentrations of CRP, an acute phase reactant, have also been seen during the systemic inflammatory response (Esmon 2005). CRP has been shown to facilitate monocyte-endothelial cell interactions (Han *et al.* 2004), and aid in the formation of PAI-1 and TF (Cermak *et al.* 1993, Devaraj *et al.* 2003). Further, CRP mediates the activation of the complement system, thereby increasing the available membrane surfaces on which initiation and amplification aspects of coagulation can proceed (Wolbink *et al.* 1998). Both platelet count and reactivity have been shown to increase in response to inflammatory mediators, such as IL-6 (Esmon 2005). In addition to increased platelet production, newly formed platelets brought about by inflammatory mediators tend to be more thrombogenic (Burstein 1997). Platelet responsive, and thus the rate of platelet thrombi generation, can also be indirectly increased by inflammatory mediators (e.g. histamine, TNF-α, IL-8 and IL-6), which lead to the release of ultra-large vWF multimers from the endothelium (Bernardo *et al*. 2004). Diminished activity of anticoagulant pathways have also been observed in response to inflammation, including decreases in antithrombin concentration and the concentration of vascular heparin-like molecules (Esmon 2005, Klein *et al*. 1992, Levi *et al.* 2004). The protein C pathway appears to be especially sensitive to down-regulation by inflammatory mediators (Esmon 2005, Levi *et al.* 2004).

In the extreme, inflammatory mechanisms that cause an exaggerated or insufficiently controlled haemostatic response, can lead to a situation in which coagulation and thrombosis become pathological. Disseminated intravascular coagulation (DIC) is one such coagulation

disorder that is considered to result from severe haemostatic imbalance, where an increase of coagulation potential occurs (Johnstone *et al.* 1986, Morris e*t al*. 1988, Welch *et al.* 1992). DIC is a pathological process characterised by the widespread intravascular activation of the coagulation cascade that results in thrombi formation in small to medium-sized blood vessels throughout the body, and ultimately leads to organ dysfunction (Taylor *et al.* 2001, Wada 2004, Wada *et al.* 2014). Various investigations have been conducted on the occurrence of DIC in colic horses (Dolente *et al.* 2002, Monreal *et al.* 2000, Stokol *et al.* 2005, Welch *et al.* 1992). Diagnosis of DIC is based on a primary condition capable of initiating DIC, clinical signs of haemorrhage or thrombosis, and irregular results of various laboratory tests of haemostasis (e.g. platelet count, coagulation assays) and fibrinolytic markers (Calverley & Liebman 2000, Dallap 2004, Morris 1988). Recently, there has been increasing interest in the use of FDPs and D-dimer as sensitive indicators of excessive fibrinolysis due to DIC (Stokol *et al.* 2005). Due to its dual role in coagulation and inflammation, changes in fibrinogen can be related to abnormalities in both processes. From the inclusion of fibrinogen in a complete coagulation panel, decreased fibrinogen with increased FDPs or D-dimers is an indication of activation and consumption of coagulation factors consistent with DIC (Benson *et al.* 2008, Dallap 2004, Dolente *et al*. 2002, Epstein & Brainard 2011, Stokol *et al.* 2005, Welch *et al*. 1992).

The incidence of DIC is further facilitated by an increase in core and peripheral body temperature associated with stress (Bouchama *et al.* 1996, Rosenthal *et al.* 1971, Strother *et al.* 1986, Weber & Blakely 1969). Stress-induced hyperthermia in mice has been extensively reviewed (Groenink *et al.* 2009, van der Heyden *et al.* 1997, Zethof *et al.* 1994). Despite the relationship between hyperthermia and haemostasis being widely acknowledged, the precise mechanisms responsible for DIC are still incompletely defined (Diehl *et al.* 2000).
As is previously discussed, a relationship of mutual causality has been suggested between a pro-inflammatory state and overproduction of cytokines, and hyperglycaemia and hyperinsulinemia (Hotamisligil *et al.* 1996, Lang *et al.* 1992, Schook *et al.* 2015, Spraker 1993). Iwasaki *et al.* (2007) have suggested that glucose is responsible for the activation of inflammation/coagulation-related protein expression, mediated in part by the generation of oxidative stress. Therefore hyperglycaemia, in addition to other risks factors (e.g. hyperinsulinemia, obesity, elevated cytokine levels), contributes to a pro-inflammatory state as well as hypercoagulability (Bruno *et al.* 1996, Festa *et al.* 1999, Iwasaki *et al.* 2007). This is as a result of the stimulatory effect of high glucose on NF-κB-dependent transcription in

hepatocytes. In response to inflammation, pro-inflammatory cytokines activate the transcription factor NF-κB in hepatic cells, leading to the immediate expression of APP (e.g. CRP, SAA), procoagulants (e.g. fibrinogen and FVIII), and fibrinolysis inhibitor PAI-1 (Begbie *et al.* 2000, Bing *et al.* 2000, Cha-Molstad *et al.* 2000, Fuller & Zhang 2001, Ruan *et al.* 2001). Although beneficial in the acute phase of inflammation (Gabay & Kushner 1999), the chronic overproduction of these proteins is known to cause hypercoagulability with resultant coagulopathies. The concurrent occurrence of hyperglycaemia, hyperinsulinemia and elevated cytokine levels has been shown clearly, in recent clinical data, to produce an increase in plasma PAI-1 and fibrinogen, leading to enhanced coagulation and impaired fibrinolysis, with a resultant increase in the risk of cardiovascular events (Pandolfi *et al.* 2001, Schneider *et al.* 1993). Furthermore, recent studies suggest that adipose tissue produces TNF-α and other cytokines (Matsuzawa *et al*. 1999), which also stimulate NF-κB-dependent transcription.

Another mechanism which has been suggested to represent an initial stage in the development of DIC is the recently introduced concept of immunothrombosis. Immunothrombosis designates an innate immune response induced by the formation of thrombi in blood vessels (Engelmann & Massberg 2013). The physiological process of immunothrombosis is initiated and maintained by fibrin generation and the local accumulation of innate immune cells (particularly monocytes and neutrophils) and platelets (Engelmann & Massberg 2013). Recent findings show that immunothrombosis in microvessels impedes the dissemination and tissue invasion of pathogens and enhances bacterial killing, thereby protecting host integrity (Massberg *et al.* 2010). Immunothrombosis represents a mechanism of intravascular antimicrobial defence, that is considered to be a consequence of the evolutionary conserved link between blood coagulation and innate immunity (Dushay 2009, Li *et al.* 2002, Loof *et al.* 2011, Theopold *et al.* 2002). Although immunothrombosis is suggested to support pathogen recognition and host defence, the dysregulation of immunothrombosis is likely to constitute a decisive biological basis for the development of thrombotic disorders, including DIC. Similar to immunothrombosis DIC, which occurs in micro vessels, is induced in response to systemic infections, and is activated and sustained by thrombosis-specific molecules (e.g. TF) (Engelmann & Massberg 2013). Therefore, DIC may be triggered when immunothrombosis becomes inundated, impairing its ability to prevent the spread of pathogens and/or damaged cells. Resulting in the unrestricted formation of microvessel thrombi and the excessive activation of inflammation, which is

further aggravated by the ability of coagulation and inflammation to potentiate each other (Esmon 2005).

1.3.3 Immobilisation

Capture and Confinement

While information on stress responses to immobilisation is limited, studies suggest that it has the potential to induce a substantial stress response and disturb normal regulatory systems, resulting in undesirable side effects (Arnemo & Caulkett 2007, Colucci *et al.* 2013, Kumar *et al.* 2002). The extent of the effects of the immobilisation is dependent on the type of drug, combination, and dosage used.

The administration of immobilising agents may produce direct and indirect effects on the functional and regulatory components of the immune response. Immobilisation has been reported to modulate the stress response by inhibiting the ACTH-stimulated production of GC (Marana *et al.* 2010, Sakai *et al.* 1995). Thus, part of the effect of immobilising agents on the immune system would be due to the immunomodulatory action of GC (Colucci *et al.* 2013). These actions include the alteration of both the count and functionality of leukocytes. Immobilising agents generally induce an increase in leukocyte counts (Cocelli *et al.* 2012, Elena *et al.* 2003, Inada *et al.* 2004). There has also been a reported depression in bactericidal activities, including: inhibition of phagocytosis and respiratory burst by neutrophils and mononuclear phagocytes (Heine *et al.* 2000, Lisowska *et al.* 2013, Rizzo *et al.* 2011), the decreased proliferation of lymphocytes (Colucci *et al.* 2013), and decreased cytotoxic activity of NK cells (Colucci *et al.* 2013, Kumar *et al.* 2002, Moudgil 1986). Opiates have also been found to decrease B-cell proliferation and antibody production (Lisowska *et al.* 2013). Further, several studies have observed that immobilisation can adversely affect both T-cell and B-cell responsiveness, as well as nonspecific host resistance mechanisms (Colucci *et al.* 2013, Kumar *et al.* 2002, Moudgil 1986, Stevenson *et al.* 1990). Immobilisation affects the inflammatory response by suppressing or releasing different cytokines (Colucci *et al.* 2013). The induced depression of the immune system is short-lived and reversible, however this depression may influence morbidity and mortality in susceptible individuals (Moudgil 1986).

Although immobilisation has been reported not to have a direct effect on haemostasis (Odoom 1995), several studies have demonstrated that opioids present in the blood decrease the viscosity of blood plasma and influence the smooth muscle of the vessels, resulting in vasodilation (Beilin *et al.* 2006, Binici *et al.* 2015, Odoom 1995, Reinhart 1995). Further, immobilisation has reportedly been associated with platelet function inhibition (Binici *et al.* 2015, Faraday 2002, Odoom 1995). However, the mechanism responsible for the inhibitory effect on platelet aggregation is unknown. The inhibition of platelet function and decreased blood viscosity serves to reduce the prevalence of thrombosis (Odoom 1995).

From the above review several hypotheses could be established. Firstly, it was proposed that HPA activity, as measured by blood cortisol and FGM excretion, would decline over time as a response to habituation to the boma conditions. Secondly, immune function, as indicated by WBC count and ROS activity, would decrease as a consequence of continued stress exposure. Thirdly, circulating levels of APP Hp and SAA would increase in response to stress. Fourthly, the APR response would enhance the coagulation potential, as screened with assays of aPTT, PT, TT and fibrinogen concentration. It was further proposed that HPA activity, immune function, APP concentrations and coagulation potential would also change in response to a 30 min immobilisation period.

2 MATERIALS AND METHODS

This study provided the opportunity to conduct a longitudinal study on the stress responses associated with repeated immobilisation and handling in impala. The extent and impact of these stressors was examined with regards to: (1) body wei ght and body condition score, (2) HPA activity, (3) ROS generating capability of whole blood, (5) secretion of APP, and (6) coagulation potential of the blood. All procedures were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (AESC 2017/10/66/B).

2.1 Experimental Design

Impala (*Aepyceros melampus*) were designated for use in a separate trial of the immobilising agent thiafentanil oxalate (A3080) that was undertaken to provide clinical data for the Medicines Control Council (MCC). The purpose of this trial was to allow thiafentanil to be registered for use in impala. Prior to the commencement of the trial, the animals were caught in the wild using mass capture techniques (Laubscher *et al*. 2015) and then confined in bomas at Ngongoni Lodge (Karino, Nelspruit; Mpumalanga). The trial protocol required that the impala be held in bomas and immobilised with different doses of thiafentanil at two-weekly intervals.

2.2 Experimental Procedure

Using mass capture techniques, 13 wild impala were obtained from a nearby game farm and transported to the Ngongoni Lodge (Karino, Nelspruit; Mpumalanga). Prior to the commencement of the trial, the animals were confined in bomas on site for a period of 14 days. Throughout the trial the impala were kept in bomas, each measuring 6 x 7 m (± 10 animals/boma) (Fig. 2.1), and provided with food (pellets and lucerne) and water *ad libitum*.

Thiafentanil (A3080, Wildlife Pharmaceuticals, White River, South Africa) was used independently as the immobilising agent. No additional sedatives or tranquilisers were used as the objective of the principle trial was to assess the optimal dose of thiafentanil for use in impala. The A3080 was formulated as a sterile injectable solution in multidose vials. The delivery system was a CO_2-powered remote injection device delivering a 3 ml air-pressurised plastic dart with a 40 mm collared needle (Dan-Inject, South Africa) to ensure a deep

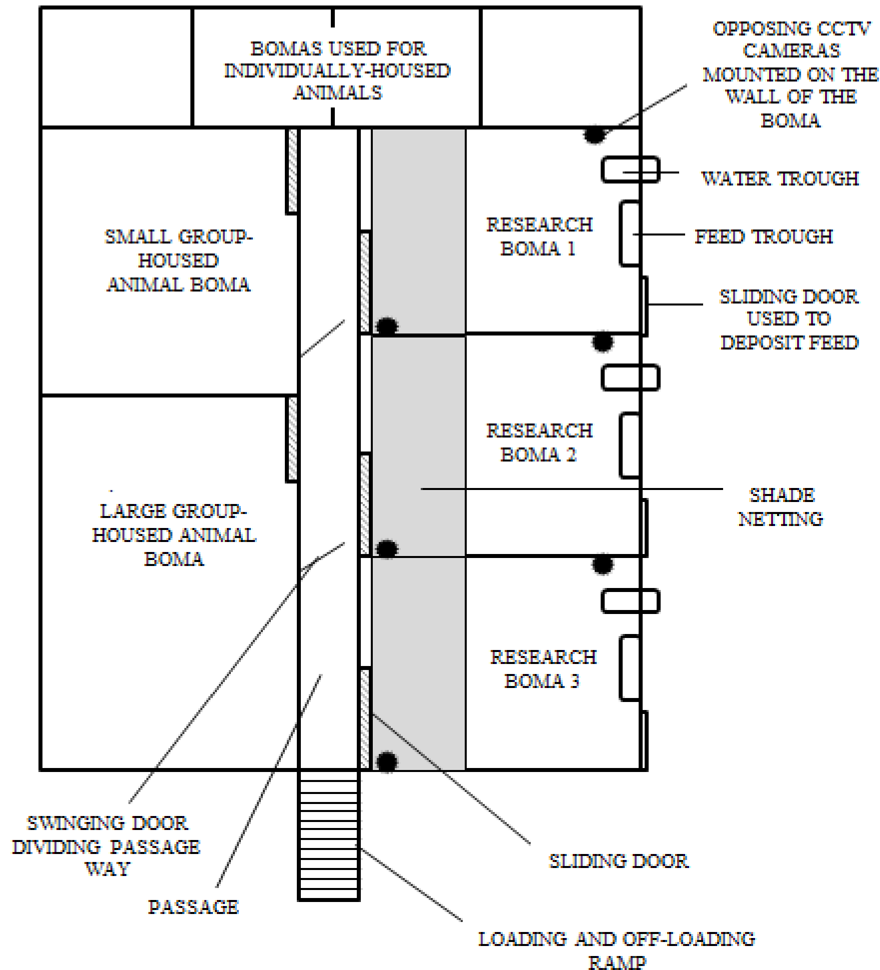


Figure 2.1 Layout of bomas on site at Ngongoni Lodge. Impala were kept in bomas each measuring 6 x 7 m (± 10 animals/boma), and provided with food and water ad libitum throughout the trial.

intramuscular injection. The impala were individually darted within the bomas at a 5 – 10 m range.

At two-weekly intervals animals were immobilised with different doses of thiafentanil (i.e. 40 $\mu g.kg^{-1}$, 80 $\mu g.kg^{-1}$ or 100 $\mu g.kg^{-1}$). To assess the effects of confinement and the response to repeated immobilisation and handling, blood samples were collected from each animal within 3-5 min of recumbency. Further, faecal samples were obtained using digital removal from the rectum. A *Cardell*® Monitor was used in Trials 3, 4 and 5 (T3, -4 and -5) to monitor all animals for a further 30 min in regards to HR, respiratory rate (RR), ECG, blood pressure (BP), oxygen saturation of arterial haemoglobin (%SpO_2), and body temperature. In addition to the *Cardell*® Monitor, parameter measurements were verified manually. Arterial blood samples were also collected from each impala to determine arterial blood gas levels, using an

i-STAT handheld blood analyser (*Abbott*®). Further measurements made on each animal at every immobilisation event included body weight (BW) and body condition. A digital hanging scale (*AE Adam*®) was used to record BW. Body condition was scored on a scale of 0-10 by subjective assessment of the tissue cover in the lumbar region (Balmford *et al.* 1992). To assess the acute effects of 30 min immobilisation we collected further blood samples from each animal after the 30 min monitoring period. Intravenous injection of naltrexone hydrochloride (naltrexone) was used to reverse the effects of immobilisation. The experimental procedure is schematically outlined in Figure 2.2.

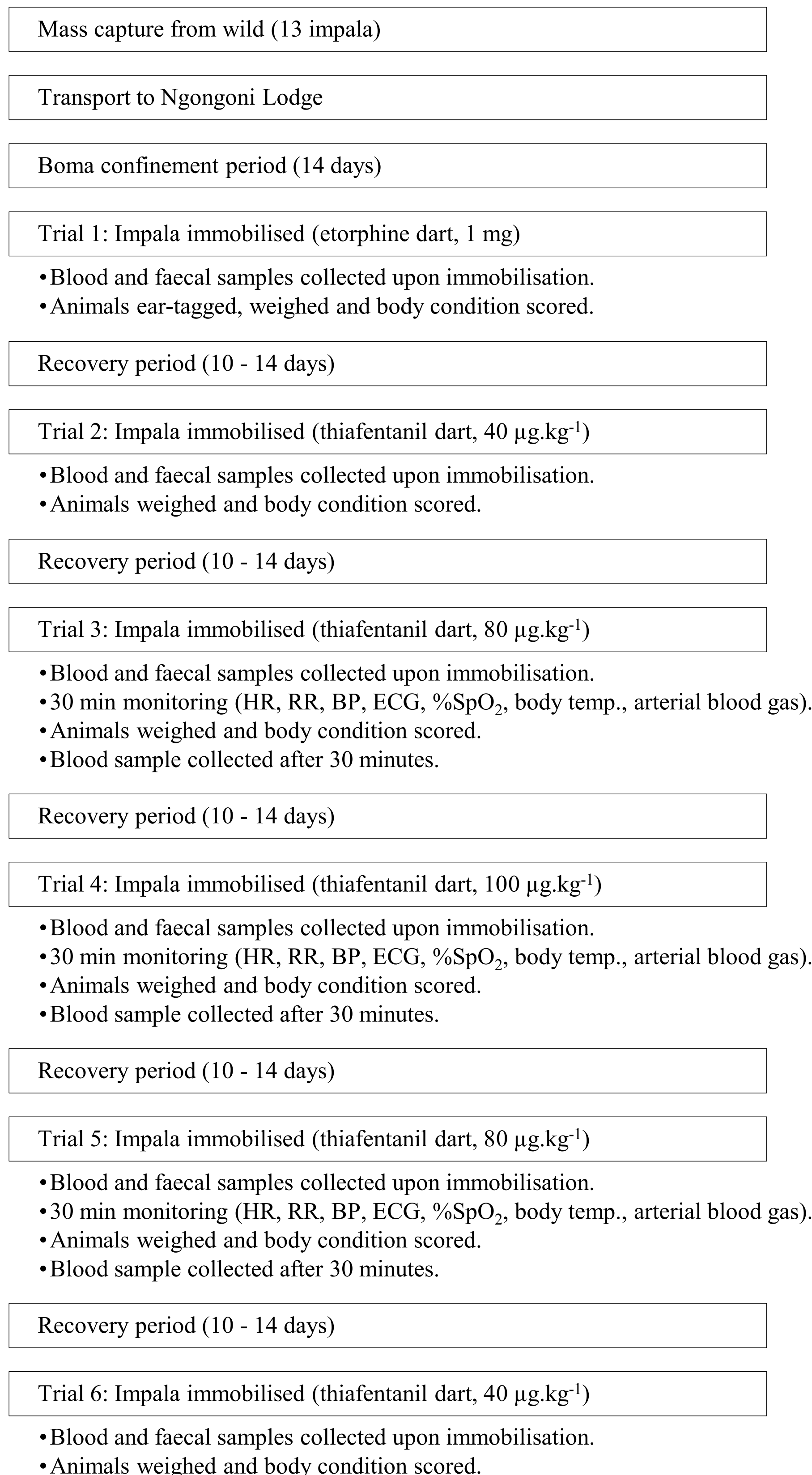


Figure 2.2 Experimental procedure outline for project conducted at Ngongoni Lodge. [HR: heart rate, RR: respiratory rate, BP: blood pressure, $\%SpO_2$: oxygen saturation of arterial haemoglobin].

2.3 Sample Collection

Venous blood samples were obtained from each animal by venipuncture of the jugular vein. At each sampling event, blood was collected into three blood collection tubes (*VACUETTE®*), namely: (1) lithium heparin (to determine hormone concentrations and innate immune activity), (2) 3.2 % sodium citrate (to determine coagulation potential), and (3) serum separator clot activator (to determine APP concentrations). The sodium citrate and serum tubes were centrifuged at 3500 rpm for 10 min each. Following the appropriate amount of whole blood being drawn out for use in determination of WBC counts and the ROS generating capability of whole blood, the lithium heparin tubes were centrifuged at 3500 rpm for 10 min each. The serum or citrated plasma from each tube was then pipetted into separate cryotubes and frozen on site at -20 °C.

Individual faecal samples were obtained from each animal by manual removal from the rectum (to determine FGM), and placed in polypropylene containers. Based on the observation that a negative relationship exists between faecal sample mass and FGM concentration (Goymann 2005, Millspaugh & Washburn 2004), we collected relatively large faecal samples (i.e. 50 ml) from each individual. Well-mixed samples were then frozen on site at -20 °C.

Following transport of the processed blood and faecal samples back to the university in Johannesburg they were stored at -80 °C and -20 °C respectively. .

2.4 Sample Processing

Measurements of HPA activity (cortisol and FGM), APP (SAA and Hp) and coagulation potential (aPTT, PT, TT and fibrinogen concentration) were done at the University of the Witwatersrand's School of Physiology on previously frozen and appropriately stored samples. Before each assay samples were thawed at room temperature. Measurements of ROS capability and WBC measurements were done in a laboratory on site at Ngongoni Lodge within 8 and 24 hours respectively after the blood sample was collected.

2.4.1 HPA Activity

To determine the HPA activity in response to capture, confinement, and immobilisation we measured:

Cortisol in Blood

Cortisol in plasma samples were measured using a commercially available Cortisol Coated Tube radioimmunoassay (RIA) kit (*Beckman Coulter, AEC-Amersham SOC Ltd)* (Stevenson 2015). Serum samples were diluted 1:10 with the manufacturer's steroid diluent and assayed according to instructions specified by the manufacturer.

Faecal Glucocorticoid Metabolites

Because FGMs may not be evenly distributed throughout the faecal mass, samples were homogenized prior to subsampling (Millspaugh & Washburn 2003, Sheriff *et al.* 2011). To eliminate variation in the moisture content between samples, a subsample of about 2.0 g (±0.001 g) was taken of each sample and oven-dried in an aluminum tin boat at 60 °C for 24 hrs. The dry faecal mass was then weighed for subsequent calculation of metabolite concentration.

The glucocorticoid metabolites in the faeces were extracted by adding 10 ml of methanol (80 %) to well-mixed 2.0 g (±0.001 g) subsamples (Möstl & Palme 1997) in 15 ml conical centrifuge tubes. Each sample was vortexed until the solution was homogenized, rotated on a vertical plane for 12 hrs and then centrifuged at 2300 rpm for 15 min. The supernatant was transferred to cryotubes and these FGM extractions were then stored at -20 °C until assayed.

Cortisol metabolites were measured in faeces using a commercially available double-antibody ^{125}I Corticosterone RIA Kit (*MP Biomedicals LLC, Thermo Fisher Scientific),* that has been validated for several species (Chinnadurai *et al.* 2009, Millspaugh & Washburn 2004, Wasser *et al.* 2000) including South African herbivores (Franceschini *et al.* 2008). Although validation and antibody specificity was not determined in this experiment, several studies have concluded that the double-antibody RIA for corticosterone shows a better performance in the detection of FGMs in different non-domestic species because of its high cross-reactivity with different metabolites (Graham & Brown 1996, Möstl & Palme 2002,

Terio *et al.* 1999, Wasser *et al.* 2000, Wielebnowski *et al.* 2002). The FGM extractions were diluted 1:10 with the manufacturer's steroid diluent and assayed according to instructions specified by the manufacturer, except for one important consideration which is a critical detail noted by my supervisor Dr. Pitts. The six corticosterone calibrators in the kit (0-1000 $ng.ml^{-1}$) are pre-diluted to account for the 1:200 dilution prescribed for rat plasma corticosterone measurement. This means that the actual concentration of each calibrator is 200 times lower than stated, so our calibration range extended from 0 $ng.ml^{-1}$ to 5 $ng.ml^{-1}$. The FGM concentrations thus determined were then corrected for dilution ratios and water content of the faeces and the results expressed as $ng.g^{-1}$ dry faeces. Serial dilutions of selected samples gave slopes of $r^2 > 0.98$ which remained parallel to the standard curve.

2.4.2 Reactive Oxygen Species Generating Capability of Whole Blood

To determine the ROS generating capability of whole blood in response to capture, confinement and immobilisation we measured:

White Blood Cell Count

The circulating level of leukocytes was measured using manual WBC counts in 1 mm^3 of blood using a modified Neubauer haemocytometer chamber (*Hawksley Crystalite*). 360 µl Turks solution was added to 40 µl whole blood in 12 x 75 mm polypropylene test tube. Turks is an acid solution that lyses erythrocytes and retains leukocytes and platelets, as well as stains the nuclei of WBCs, allowing for easy identification and counting. Turks solution was prepared by adding 3 ml glacial acetic acid, 1 ml aqueous gentian violet and 96 ml distilled water. Tubes were capped and mixed by inversion for 2 min. A cover slip was placed over the Neubauer chamber, which was then loaded with an appropriate amount of mixture by way of capillary action. The fully loaded haemocytometer was incubated at room temperature for 3 min to allow cells to settle. Under 10x magnification WBCs were then counted and recorded.

Reactive Oxygen Species Generation

We examined the capacity of leukocytes to produce a respiratory burst after a stressful event by measuring stimulated chemiluminescence levels in whole blood *in vitro* using PMA

(Fandos Esteruelas *et al.* 2016, McLaren *et al.* 2003). To measure an individual animal's LCC response three 12 x 75mm polypropylene test tubes were prepared for each sample. To each tube containing 500 µl of phosphate buffered saline (PBS), 100 µl of heparinised whole blood was added, to which 100 µl of luminol (5-amino-2.3-dihydrophthalzine) was added. Tubes were mixed by gentle shaking. Luminol chemiluminesces produce a low intensity light reaction when combined with an oxidising agent (Whitehead *et al*. 1992). All tubes were incubated at 37 °C for 5 min. Following incubation, 100 µl of PMA at a concentration of 10^{-5} mol L^{-1} was added to two tubes. The PMA solution was prepared in advance by diluting 5 mg of PMA in 500 ml of dimethyl sulfoxide, which was then diluted to a concentration of 10^{-5} mol L^{-1} in PBS buffer. The third tube served as an unstimulated control and the PMA was substituted with 100 µl of PBS. For each tube, we measured and recorded chemiluminescence in relative light units (RLU) using a portable single tube luminometer (*UNI-LITE NG2, Biotrace*®) every 5 min for a total of 45 min. When not in the luminometer, tubes were continually incubated at 37 °C.

2.4.3 Secretion of Acute Phase Proteins

To determine the secretion of APP in response to capture, confinement and immobilisation we measured:

Haptoglobin

The circulating concentrations of Hp in the impala were examined using a commercially available, colorimetric method based on peroxidase activity (*Tridelta Development Ltd.*) (Cooke & Arthington 2013, Wells *et al.* 2013). Haptoglobin can be quantitated, based on its universal reactivity with hemoglobin (Petersen *et al*. 2004, Tecles *et al.* 2007). Validation of this cross species assay for Hp was described in Crawford et al. (2013). Serum samples were assayed according to instructions specified by the manufacturer.

Serum Amyloid A

The circulating levels of SAA in the impala were examined using a commercially available, multi species enzyme-linked immunosorbent assay (ELISA) (*Tridelta Development Ltd.*) (Bernal *et al.* 2011, Petersen *et al.* 2001, Piñeiro *et al.* 2007, Wells *et al.* 2013). Serum

amyloid A was quantitated as it appears to be conserved within mammalian species (Hol & Gruys 1984, Jacobsen *et al.* 2006) and it has also been reported to be a likely major APP in non-domesticated mammals (Stanton *et al*. 2013). Serum samples were diluted 1:10 with steroid diluent and assayed according to instructions specified by the manufacturer.

2.4.4 Coagulation Potential

To determine the coagulation potential in response to capture, confinement and immobilisation we measured aPTT, PT, TT and fibrinogen concentration using the manual tilt-tube method (Bai *et al.* 2008, D'Angelo *et al.* 1989). After adding factors to promote activation of the relevant clotting cascade, tubes were tilted regularly back and forth against a light source until the first fibrin clot was visible. Clotting time was recorded in seconds. Duplicate determinations were made for each sample.

Activated Partial Thromboplastin Time

We examined the activity levels of the extrinsic pathway (including factors II, V, VII, X, and fibrinogen) using an activated partial thromboplastin time (aPTT) assay (*Randox Laboratories Ltd*) (Benson *et al.* 2008, Hussein *et al.* 2010, Siroka *et al.* 2011). The aPTT reagent was pre-warmed at 37 °C. 0.1 ml of each sample was transferred to a 12 x 75 mm polypropylene test tube, to which 0.1 ml of pre-warmed aPTT reagent was added. Tubes were incubated at 37 °C for 3 min. We added 0.1 ml of calcium chloride reagent to each tube singly, and started timer simultaneously.

Prothrombin Time

The activity levels of the intrinsic pathway (including factors II, V, VIII, IX, X, XI, XII, and fibrinogen) were examined using a prothrombin time (PT) assay (*Randox Laboratories Ltd*) (Benson *et al.* 2008, Hussein *et al.* 2010, Siroka *et al.* 2011). The PT reagent was pre-warmed at 37 °C. 0.1 ml of each sample was transferred to a 12 x 75 mm polypropylene test tube, and pre-warmed at 37 °C for 2 min. We added 0.2 ml of pre-warmed PT reagent to each tube singly, and started timer simultaneously.

Thrombin Time

The rate of fibrinogen conversion was examined using a thrombin time (TT) assay (*Randox Laboratories Ltd*) (Benson *et al.* 2008, Hussein *et al.* 2010, Siroka *et al.* 2011). We transferred 0.2 ml of each sample to a 12 x 75 mm polypropylene test tube, and pre-warmed at 37 °C for 3 min. We added 0.1 ml of reconstituted thrombin time reagent to each tube singly, and started timer simultaneously.

Fibrinogen

The fibrinogen concentration was determined using a modified Clauss method (*Randox Laboratories Ltd*) (Benson *et al.* 2008, Hussein *et al.* 2010, Siroka *et al.* 2011). In the presence of a high concentration of thrombin the time required for formation of the clot in diluted plasma is inversely proportional to the fibrinogen concentration. We diluted 0.1 ml of controls and samples in 0.9 ml of imidazole buffered saline. In 12 x 75 mm polypropylene test tubes, 0.2 ml of each dilution was incubated at 37 °C for 1 min. We added 0.1 ml of reconstituted thrombin reagent to each tube singly, and started timer simultaneously.

2.5 Data Processing and Statistical Analysis

Data was analysed using the GraphPad Prism (Version 5.0.4) statistical software package (La Jolla, California, USA). The significance level for all comparisons was established as $P < 0.05$, and all testing was two-tailed. Normality testing was conducted to assess if all parameters were normally distributed. To analyse the chronic effects of confinement and response to repeated stressors, repeated measures one-way analyses of variance (ANOVAs) were conducted for each variable that was normally distributed. Bonferroni post hoc tests were conducted on column data indicated to be parametric. For non-parametric data the Friedman statistic was calculated and Dunn's tests used to compare columns. Data are expressed as box plots with median and 95% confidence levels. Upper and lower limits of the box represent the 75% and 25% percentiles respectively. To analyse the response to 30 min of immobilisation paired t-tests were conducted for each parameter. Data are expressed as mean ± SD (standard deviation).

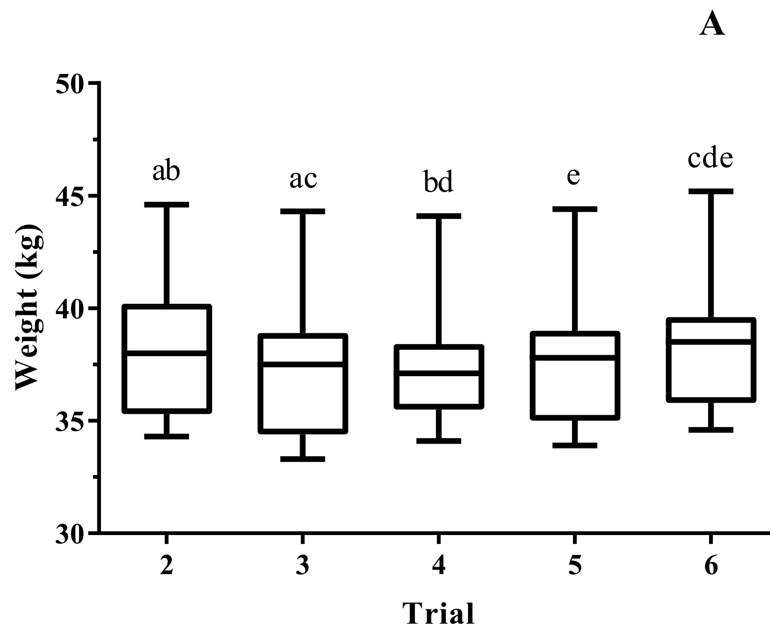
3 RESULTS

3.1 Confinement and Repeated Immobilisation

The following are the results obtained from the blood and faecal samples collected from each animal upon immobilisation, for the purpose of assessing the effects of confinement and the response to repeated immobilisation and handling.

3.1.1 Body Weight and Condition

In response to confinement and repeated immobilisation we observed a significant decline in body weight (Fig. 3.1A) in the impala from T2 to T3 ($P \leq 0.05$) and T4 ($P \leq 0.01$). Thereafter, a significant increase in body weight was observed at T6, such that the animals had returned to their initial average body weight at T1. Concurrently, we observed a significant decrease in body condition (Fig. 3.1B) from T2 to T3 ($P \leq 0.01$) and T4 ($P \leq 0.01$), followed by a significant increase in body condition at T6, which corresponds with the recovery of body weight to the initial conditions.



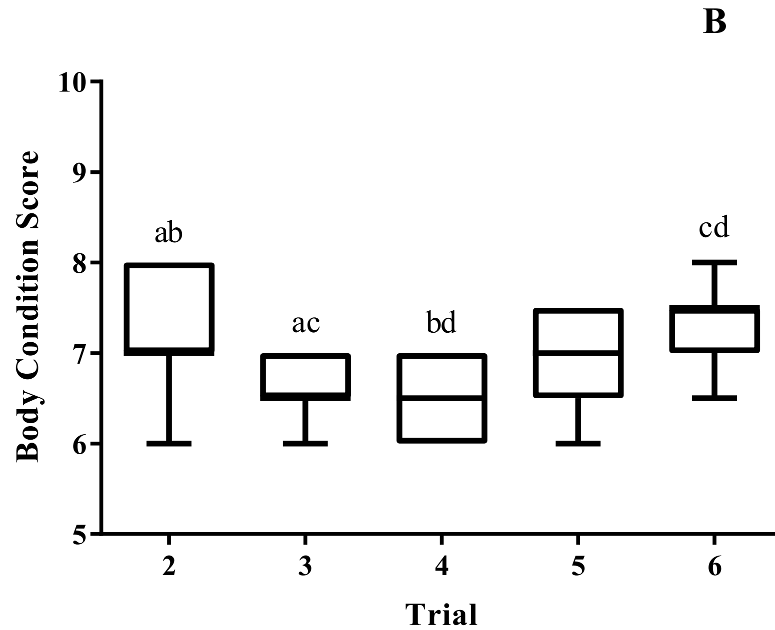


Figure 3.1 Body weight and condition in response to confinement and repeated immobilisation in impala. (A) Impala body weight, and (B) impala body condition. Data are expressed as box plots with median and 95% confidence levels. Upper and lower limits of the box represent the 75% and 25% percentiles respectively. Significant difference ($P < 0.05$) between immobilisation events was determined using one-way ANOVA followed by Bonferroni post hoc tests. Columns in either A or B with the same letter (a, b, c, d or e) are significantly different from each other respectively.

The health characteristics of the 11 impala that completed our trials suggest that our sample was in reasonably good health. Subjects that did not complete the trials included two animals that were euthanised due to a broken leg and fetlock respectively, sustained during the boma darting procedures T4.

3.1.2 HPA Activity

Cortisol in Blood

No significant difference in cortisol concentration in response to darting after a period confinement and repeated immobilisation was observed in the impala ($P = 0.056$).

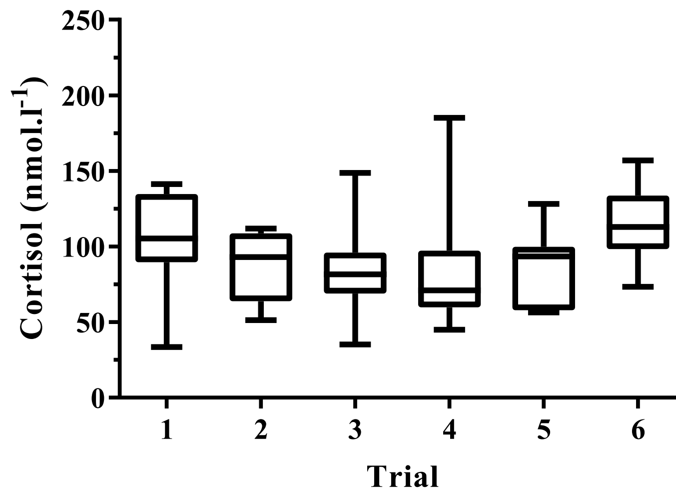


Figure 3.2 Cortisol response to confinement and repeated immobilisation in impala. Data are expressed as box plots with median and 95% confidence levels. Upper and lower limits of the box represent the 75% and 25% percentiles respectively. Significant difference ($P < 0.05$) between immobilisation events was determined using one-way ANOVA followed by Bonferroni post hoc tests. No significance was observed ($P = 0.056$).

Faecal Glucocorticoid Metabolites

In the impala we observed a significant increase in FGM concentration from T1 to T3 ($P \leq 0.01$) in response to confinement and repeated immobilisation. Thereafter the FGM concentration declined to below that which was recorded at T2 and T3.

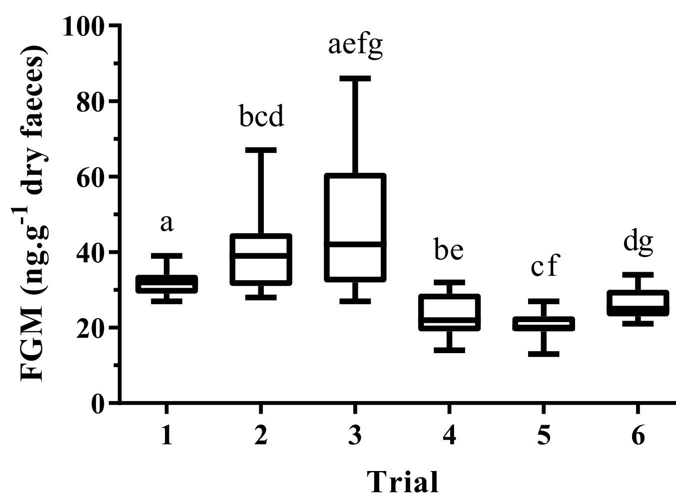


Figure 3.3 Faecal glucocorticoid metabolite responses to confinement and repeated immobilisation in impala. Data are expressed as box plots with median and 95% confidence levels. Upper and lower limits of the box represent the 75% and 25% percentiles respectively. Significant difference ($P < 0.05$) between immobilisation events was determined using one-way ANOVA followed by Bonferroni post hoc tests. Columns with the same letter (a, b, c, d, e, f or g) are significantly different from each other respectively.

3.1.3 Reactive Oxygen Species Generating Capability of Whole Blood

White Blood Cell Count

No significant difference in WBC count was observed in response to confinement and repeated immobilisation in the impala ($P = 0.4066$).

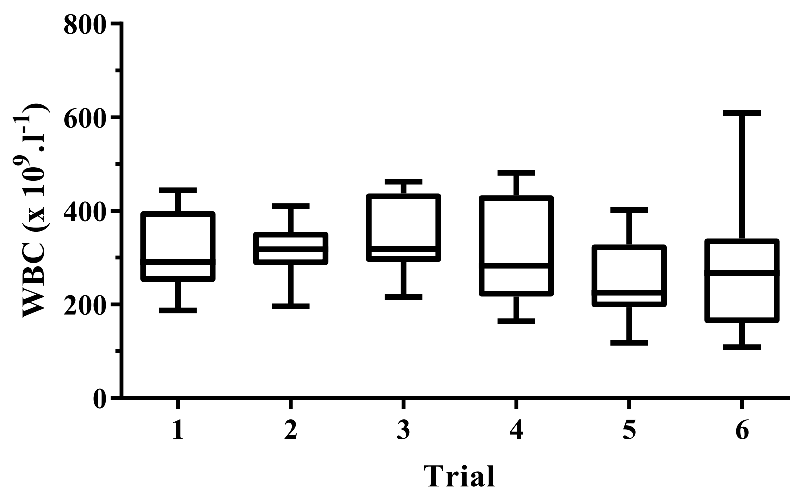


Figure 3.4 White blood cell responses to confinement and repeated immobilisation in impala. Data are expressed as box plots with median and 95% confidence levels. Upper and lower limits of the box represent the 75% and 25% percentiles respectively. Significant difference ($P < 0.05$) between immobilisation events was determined using one-way ANOVA followed by Bonferroni post hoc tests. No significance was observed ($P = 0.4066$).

Reactive Oxygen Species Assay

In response to confinement and repeated immobilisation a significant decline in leukocyte respiratory burst activity in the impala was observed from T2 to T4 ($P \leq 0.05$). This was followed by a significant increase from T4 to T6 ($P \leq 0.05$), which showed recovery to initial

levels. The decline in ROS generating capability in the absence of a significant decline in WBC count indicates that there is a functional decline in these cells.

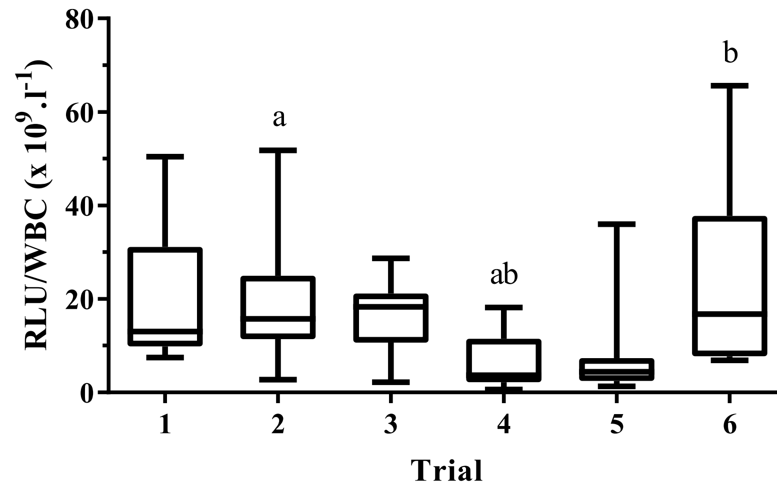


Figure 3.5 Reactive oxygen species generating capability of whole blood in response to confinement and repeated immobilisation in impala. Data are expressed as box plots with median and 95% confidence levels. Upper and lower limits of the box represent the 75% and 25% percentiles respectively. Significant difference ($P < 0.05$) between immobilisation events was determined using one-way ANOVA, followed by calculation of Friedman statistic and Dunn's post hoc tests. Columns with the same letter (a or b) are significantly different from each other.

3.1.4 Secretion of Acute Phase Proteins

In the impala no significant change in either the Hp or SAA concentrations in response to confinement and repeated immobilisation was observed, although increasing variability in the results was noted ($P = 0.1354$ and $P = 0.167$ for Hp and SAA respectively) (Fig. 3.6).

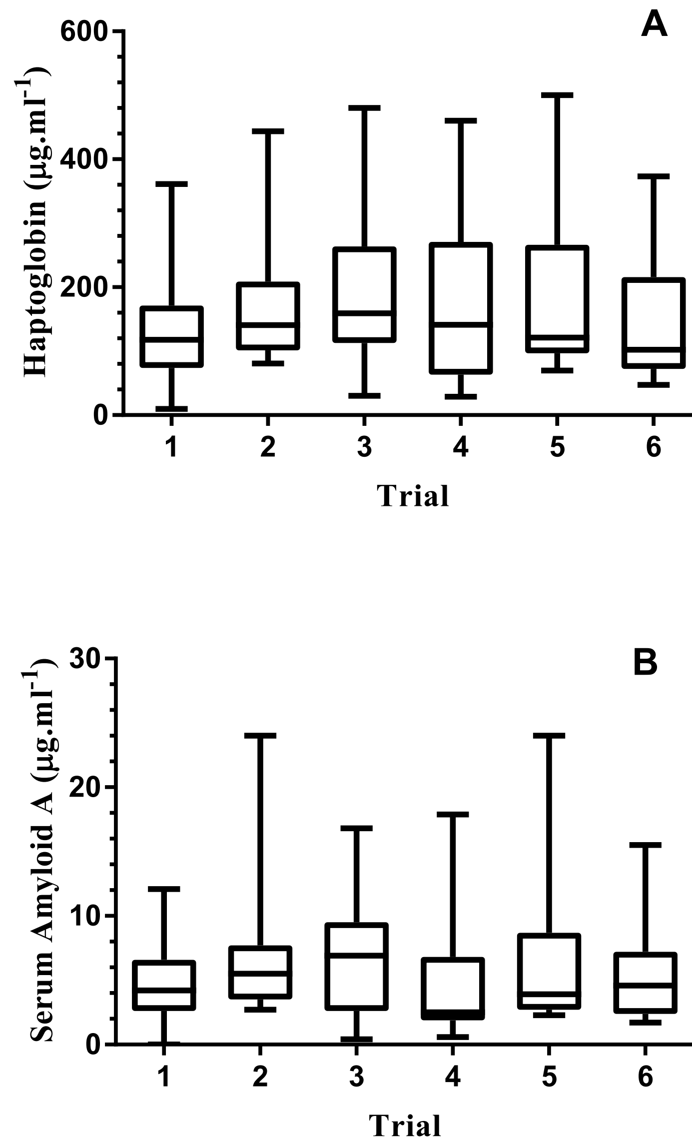


Figure 3.6 Secretion of acute phase protein responses to stress in the impala. (A) Haptoglobin concentration in blood, and (B) serum amyloid A concentration in blood. Data are expressed as box plots with median and 95% confidence levels. Upper and lower limits of the box represent the 75% and 25% percentiles respectively. Significant difference ($P < 0.05$) between immobilisation events was determined using one-way ANOVA, followed by calculation of Friedman statistic and Dunn's post hoc tests. No significance was observed (Friedman $P = 0.1354$ and $P = 0.167$ for A and B respectively).

3.1.5 Coagulation Potential

Figures 3.7 show the effects of confinement and repeated immobilisation on aPTT, PT, TT and fibrinogen concentration in the impala.

Activated Partial Thromboplastin Time

Activation of the intrinsic pathway, as measured by the aPTT, was noted to increase at T4 (Fig. 3.7A). Thereafter there was a significant decline in the clotting time from T4 to T5 ($P \leq 0.05$) and T6 ($P \leq 0.01$), which showed recovery to initial levels.

Prothrombin Time

In the impala a significant delay in activation of the extrinsic pathway was seen as an increase in PT was observed from T1 to T3 ($P \leq 0.001$) (Fig. 3.7B). From T4 to T6 the clotting time showed recovery to initial levels ($P \leq 0.01$).

Thrombin Time

The conversion rate of fibrinogen to the fibrin clot, as indicated by the TT assay, decreased significantly at T3 compared to levels at T1 ($P \leq 0.001$) and T2 ($P \leq 0.001$) (Fig. 3.7C). Thereafter the TT again increased over successive trials until it showed recovery to initial levels.

Fibrinogen Concentration

A significant increase in fibrinogen concentration was observed at T4 compared to levels at T1 ($P \leq 0.05$) and T3 ($P \leq 0.01$) (Fig. 3.7D). Thereafter, a significant decline was observed at T6 ($P \leq 0.01$), which showed recovery to initials levels.

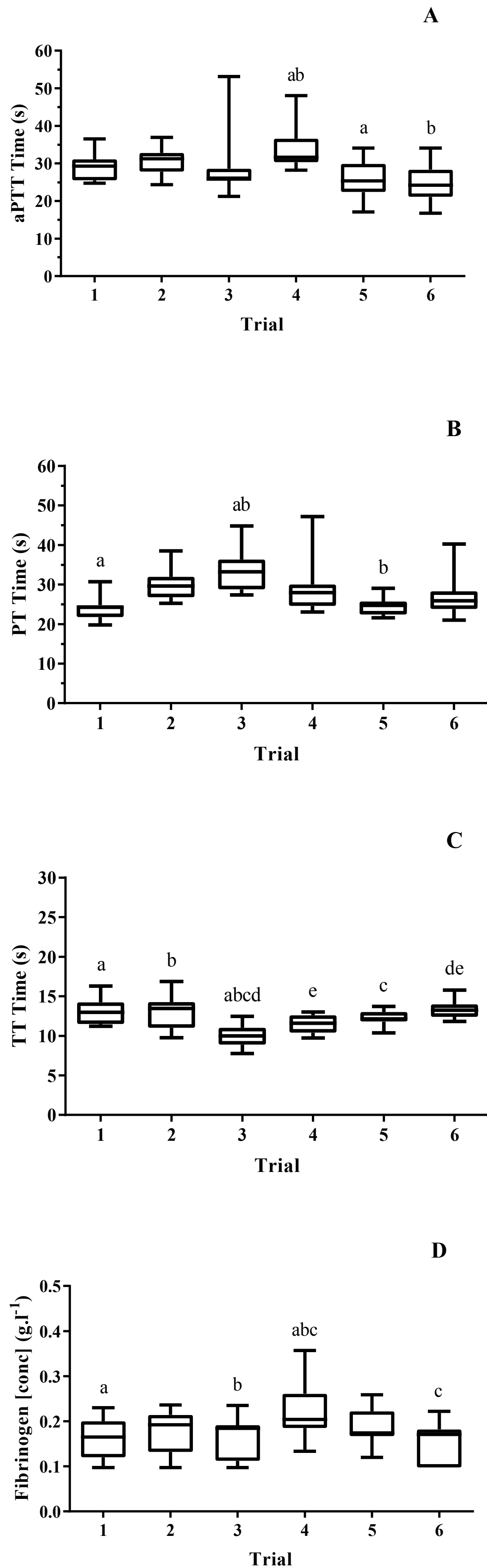


Figure 3.7 Coagulation potential responses to confinement and repeated immobilisation in impala. (A) Impala activated partial thromboplastin time, (B) prothrombin time, (C) thrombin time, and (D) fibrinogen concentration. Data are expressed as box plots with median and 95% confidence levels. Upper and lower limits of the box represent the 75% and 25% percentiles respectively. Significant difference ($P < 0.05$) between immobilisation events was determined using one-way ANOVA followed by Bonferroni post hoc tests. Columns in each block with the same letter (a, b, c, d or e) are significantly different from each other respectively.

3.2 30 Minutes of Immobilisation

The following are the results obtained from the blood samples collected from each animal following the 30 min monitoring period (at T3, -4 and -5), for the purpose of assessing the acute effects of 30 min of immobilisation.

3.2.1 HPA Activity

Cortisol in Blood

As shown in Table 3.1 the cortisol concentrations in the impala significantly increased at T3 ($P = 0.009$) and T5 ($P = 0.0323$) in response to 30 min of immobilisation.

3.2.2 Reactive Oxygen Species Generating Capability of Whole Blood

White Blood Cell Count

No significant change in WBC count as a consequence of 30 min of immobilisation with thiafentanil was observed in the impala (Tab. 3.1).

Reactive Oxygen Species Assay

The ROS generating capability of whole blood in the impala showed a significant decrease over the 30 min of immobilisation at T4 ($P = 0.0406$) and T5 ($P = 0.0188$) (Tab. 3.1).

3.2.3 Secretion of Acute Phase Proteins

SAA and Hp were only measured at T4. No significant difference in concentration for either APP was observed after 30 min of immobilisation.

3.2.4 Coagulation Potential

aPTT and PT did not change significantly as a result of the 30 min of immobilisation. We observed a significant decrease in TT during T3 ($P = 0.0002$), T4 ($P < 0.0001$) and T5 ($P =$

0.0020) as a consequence of 30 min of immobilisation. The fibrinogen concentration decreased in response to 30 min of immobilisation at T3 (*P* = 0.0457) (Tab. 3.1).

Table 3.1 HPA activity, reactive oxygen species generating capability of whole blood, and coagulation potential responses to immobilisation by thiafentanil. Data are expressed as mean ± SD (standard deviation). Significant difference ($P < 0.05$) between initial immobilisation and after 30 min of immobilisation was determined for each parameter within each trial using paired t-tests. [WBC: white blood cell, RLU: reactive light unit, aPTT: activated partial thromboplastin time, PT: prothrombin time, TT: thrombin time].

	Trial 3			Trial 4			Trial 5		
Parameter	Initial Immobilisation (0 min)	30 min after Immobilisation	*P* value	Initial Immobilisation (0 min)	30 min after Immobilisation	*P* value	Initial Immobilisation (0 min)	30 min after Immobilisation	*P* value
Cortisol ($nmol.l^{-1}$)	87.43 ± 31.41	51.65 ± 37.59	0.0009	83.37 ± 38.27	61.25 ± 45.09	0.1523	87.15 ± 23.13	59.55 ± 35.01	0.0323
WBC (x $10^9.l^{-1}$)	342.5 ± 79.30	298.90 ± 61.09	0.1438	314.4 ± 113.6	295.2 ± 141.4	0.4633	259.40 ± 92.81	271.50 ± 99.06	0.5287
$RLU.WBC^{-1}$ (x $10^9.l^{-1}$)	16.20 ± 8.68	10.40 ± 8.56	0.1031	6.071 ± 5.62	8.80 ± 7.66	0.0406	7.11 ± 9.79	12.46 ± 15.42	0.0188
aPTT (s)	29.27 ± 9.31	30.18 ± 3.39	0.7793	33.75 ± 5.55	32.29 ± 4.47	0.4410	25.73 ± 5.11	25.73 ± 5.33	0.9997
PT (s)	33.57 ± 6.03	31.56 ± 3.02	0.4133	29.25 ± 6.49	26.02 ± 1.76	0.1096	24.55 ± 2.21	24.59 ± 2.390	0.9487
TT (s)	9.96 ± 1.44	10.72 ± 1.46	0.0002	11.44 ± 1.12	13.48 ± 0.80	< 0.0001	12.33 ± 1.09	13.42 ± 1.05	0.0020
Fibrinogen ($mg.l^{-1}$)	161.40 ± 50.05	188.60 ± 48.49	0.0457	225.60 ± 60.65	208.60 ± 55.44	0.3298	183.60 ± 38.29	168.80 ± 51.21	0.1124

4 DISCUSSION

The primary objective of this study was to improve the understanding of the impact of capture, confinement and immobilisation on specific physiological systems in impala. Impala are ideal for studying the responses of animals to events associated with these stressors due to the availability of data from other studies conducted on impala, and other similar ungulate species, which have included appropriate physiological measures (Cross *et al.* 1988, Delgiudice *et al.* 1990, Hattingh *et al.* 1988, Kock *et* al. 1987, Kock *et al.* 1990, Knox *et al.* 1992, Peinado *et al.* 1993, Peinado *et al.* 1995, Morton *et al.* 1995, Wesson *et al.* 1979). In addition, their responsive nature and susceptibility to stress-related diseases, allow impala to act as sensitive indicators of events that might also elicit a reaction from other wild, semi-wild, and domestic animals.

With respect to the measurements made at the beginning of each new trial, body weight and condition of the animals declined significantly until T4, with recovery thereafter. Plasma cortisol did not show any change with the stress of repeated immobilisation, but the FGM results indicate that an increase in HPA activity did occur up to T3, but then declined to levels below that recorded at T1. Total WBC counts showed no change during the duration of the study, but the ability of whole blood to produce ROS when stimulated to do so declined significantly by T4 and T5. Concentrations of the circulating APR did not change over the course of the study, but the concentration of the blood protein fibrinogen increased significantly by T4. This increase resulted in a significant increase activity of the common coagulation pathway (as indicated by the decrease in the TT). The coagulation pathways measured by aPTT and PT were significantly slowed up to T3 and T4, but then recovered as body weight and condition improved.

Throughout the duration of the present study the impala were provided with highly palatable food (pellets and Lucerne) *ad libitum*. Despite this fact, there was a significant decline in the average body weight and condition in the impala from T2 to T4. The body weight and condition subsequently recovered to initial levels observed in the impala. This phenomenon of recovery can perhaps be attributed to this species exhibiting habituation to confinement and repeated immobilisation. As was noted in Chapter 1, an organism adaptively responds to adversity by activating the neuroendocrine axis which results in the increased immediate availability of energy, to up-regulate physiological processes that are required for survival

(Creel *et al.* 2001, Dantzer *et al*. 2014, Guyre *et al.*1984, Sapolsky 2002). This response involves catecholamine-induced lipolysis and gluconeogenesis, and redirection of resources by circulating GC, thereby facilitating the mobilisation of energy sources to the CNS and somatic muscle (Creel *et al.* 2001, Dickens *et al*. 2010, Spraker 1993). In chronically stressed individuals a loss of muscle mass has been observed (Creel *et al.* 2001) as a result of the reduced efficiency of the negative feedback mechanism to suppress the HPA axis once the stressor diminishes or ceases (McEwen & Seeman 1999, Romero *et al.* 2009, Sapolsky *et al.* 1984). Body weight and condition can therefore be expected to decline in response to prolonged stress exposure and can be considered as a standard biomarker of chronic stress (Dickens & Romero 2013).

Glucocorticoids are considered classic mediators of the physiological stress response, and are fundamental to how animals integrate, respond and adapt to both predictable and unpredictable perturbations in their environment (Creel 2001, Dantzer *et al.* 2014). In the present study, the plasma cortisol concentration obtained at each immobilisation never differed significantly from the values obtained at the first immobilisation. In a similar study by Hattingh *et al.* (1988) the plasma cortiol concentration in blood obtained by manually restraining boma-kept impala, was compared to control values from headshot animals in the wild. Cortisol values were significantly greater in boma-kept animals (93 ± 21 $nmol.l^{-1}$) than mean control values obtained for impala (19 ± 8 $nmol.l^{-1}$) (Hattingh *et al.* 1988, Hattingh *et al.* 1990). Additonal repeated capture and handling of the boma-kept impala over a prolonged period of confinement resulted in statistically insignificant changes in cortisol level (Hattingh *et al.* 1988, Hattingh *et al.* 1990). In agreement with this, we observed high blood cortisol levels in the impala which remained unchanged across all trials. These consistently elevated values can perhaps be attributed to the impala associating the presence of humans with discomfort, resulting in an anticipatory, conditioned response to the immobilisation procedure (Hattingh *et al.* 1988, Hattingh *et al.* 1990, Ramade & Baylé 1984). Inherent in such a reaction would be the animals mounting a hormonal stress response already prior to immobilisation at each capture event (Ramade & Baylé 1984). Due to the tendency of stress metrics, especially measurements taken from blood, to change rapidly and unpredictably (Dickens & Romero 2013, Lynn & Porter 2008, McLaren *et al.* 2003, Romero & Romero 2002, Wingfield *et al.* 1982) this anticipatory response may result in a biased representation of cortisol secretion in response to the chronic effects of confinement and repeated

immobilisation, and possible adaptation. We suggest that cortisol in blood is, therefore, not a good indicator of stress in the assessment of welfare during conservation management.

To overcome the inherent limitations associated with measuring GC in blood, we also used the noninvasive technique of measuring GC metabolites excreted in the faeces (Millspaugh & Washburn 2004). FGM measurement provides a more stable and accurate assessment of stress without the bias of capture-induced increases in GC (Harper & Austad 2000, Millspaugh *et al*. 2001, Touma *et al*. 2003). Furthermore, FGM assays reflect an integrated average of blood GC, and may therefore more closely reflect the cumulative exposure of individuals to GC, rather than a point sample, providing a more accurate assessment of long-term GC levels (Goymann 2005, Harper & Austad 2000, Hirschenhauser *et al.* 2005, Ninnes *et al.* 2010, Sheriff *et al*. 2011). During an acute stress-induced response, circulating GC levels typically increase and primarily function to ensure the immediate availability of energy that can be used to resolve stressful situations by redirecting resources and mobilising body reserves (Chinnadurai *et al.* 2009, Creel 2001, Teixeira *et al.* 2007). In the current study, the general tendency of the FGM concentration in the impala to significantly increase from T1 to T3 is consistent with a decline in body weight and condition. These results are indicative of increased HPA activity in response to the chronic effects of confinement and repeated stressors, and the resultant depletion of body reserves (Dickens & Romero 2013, Sapolsky 2002, Sapolsky *et al.* 1986). When analysing FGM, the concentration of hormone metabolites is indicative of the cumulative concentration of circulating hormones that have been secreted over a range of time (Goymann 2005, Hirschenhauser *et al.* 2005, Young & Hallford 2013). The subsequent significant decline in FGM levels observed in the impala may, therefore, be attributed to the inability of the HPA axis to maintain a response over a 24 hr period. As the trials progressed, however, a significant restoration in body weight and condition was observed in the impala. Subsequently a restoration in FGM concentration was observed, to the point that FGM levels were lower than at T1. This observation suggests that the animals required less daily HPA secretion to survive, which is possibly due to the fact that (i) high quality feed was available and (ii) the animals were becoming habituated to the boma confinement.

The results obtained in the present study confirm that the MP Biomedicals ^{125}I Corticosterone RIA is capable of measuring physiologically relevant changes in FGM levels in the impala. Our study supports the findings of other studies that have used this assay and shown FGM

analysis to be an efficient means of quantifying physiological stress in ungulate species (Chinnadurai *et al.* 2009, Millspaugh *et al.* 2002, Millspaugh *et al.* 2001, Wasser *et al.* 2000).

Stress affects the immune system by altering the composition, quantity, activity and responsiveness of circulating immune cells (Dhabhar *et al.* 1995, Ellard *et al.* 2001). Leukocyte counts represent a particularly useful indicator of the characteristic changes elicited by increased GC hormones on the vertebrate immune system (Davis *et al.* 2008). Upon exposure to stress hormones a marked alteration in leukocyte count has consistently been observed in mammalian species (Dhabhar *et al*. 1995, Dougherty & White 1944, Gordon 1955). In the present study, the absence of a significant change in WBC observed in the impala can perhaps be attributed to stress-induced changes on leukocyte profiles, namely the increases in neutrophil numbers (neutrophilia) and decreases in numbers of lymphocytes (lymphopenia) (Davis *et al.* 2008). In response to external stimuli, GC stimulate the influx of neutrophils into the blood from bone marrow, which aid in an individual's ability to mount an immune response by releasing ROS (Davis *et al.* 2008, Ellard *et al.* 2001, Fandos Esteruelas *et al.* 2016, Montes *et al.* 2004). In contrast, circulating lymphocyte numbers are reduced by glucocorticoid-induced alterations in the redistribution of lymphocytes from the blood to other body compartments (e.g. lymph nodes, skin), to help mount an immune response (Davis *et al.* 2008, Dhabhar 2002). Extensive literature exists on the phenomenon of stress-induced neutrophilia and lymphopenia in a wide range of mammalian species (Dougherty & White 1944, Frank *et al.* 2006, Gordon 1955, Kock *et al.* 1999, Noda *et al.* 2007, Obernier & Baldwin 2006).

Recently, ecological studies have shown the significant impact of oxidative status on fitness components in wild animals (Beaulieu & Costantini 2014, Fandos Esteruelas *et al.* 2016, McLaren *et al.* 2003). ROS are produced as part of the normal aerobic metabolism of mitochondria (Beaulieu & Costantini 2014). In response to a stressor, leukocytes (in particular neutrophils) are activated and enhance the release of ROS via a process called respiratory burst (Ellard *et al.* 2001, Montes *et al.* 2004). However, prolonged secretion of stress hormones has consistently been observed to reduce the ability of leukocytes to produce oxygen free radicals in response to stimulation (Fandos Esteruelas *et al.* 2016, Gelling *et al.* 2009, Honess *et al.* 2005, McLaren *et al.* 2003, Moorhouse *et al.* 2007, Valle *et al.* 2015). The significant decline in ROS production observed in the current study is consistent with the supporting literature. Additionally, the results obtained support the reinterpretation of stress-

immune interactions proposed by Dhabhar and McEwen (1997, 2001), wherein chronic stress suppresses the immune response (Martin 2009, Segerstrom & Miller 2006). Since the ROS data is expressed relative to the number of circulating leukocytes, the data shows that capacity of whole blood to produce ROS is compromised. This result contradicts those of Fandos Esteruelas *et al.* (2016) and McLaren *et al.* (2003), who could attribute a decline in ROS production to a decline in leukocyte numbers. The significant increase in ROS production observed in the final trial (i.e. T6) of our study can perhaps be attributed to leukocyte reactivity being reestablished once the daily HPA activity, as shown by the FGM levels, began to decline. Furthermore, the initial decline and subsequent restoration of ROS production seen in the impala corresponds with the pattern of results observed for FGM concentration, and body weight and condition in this species, indicating the applicability of measuring LCC to assess the welfare of these animals.

Nonspecific markers of systemic inflammation, APP, have been shown to provide an added sensitivity over traditional methods of assessing inflammation due to their rapid and high magnitude increase and short half-life (Cray *et al.* 2009, Eckersall & Bell 2010, Kjelgaard-Hansen & Jacobsen 2011, Stanton *et al.* 2013). Recently, several studies have demonstrated the utility of APP quantitation in monitoring neoplasia and stress (Ceciliani *et al.* 2012, Cray *et al.* 2009, Piñeiro *et al.* 2007). In the present study acute phase proteins SAA and Hp were quantitated for the purpose of monitoring the APR. Serum amyloid A appears to be conserved within mammalian species and Hp can be quantitated based on its universal reactivity with haemoglobin (Hol & Gruys 1984, Jacobsen *et al.* 2006). Data from the present study demonstrates that the ability to mobilise these proteins is maintained in spite of the effects of acute and chronic stress, indicating the importance of preserving this response in the face of physiological adversity.

The accumulation of several studies has resulted in the emergence of a fairly reliable coagulation and fibrinolysis pattern in response to stress exposure (von Känel 2015, von Känel *et al.* 2001a). A haemostatic equilibrium has been observed in healthy individuals between thrombosis and haemorrhage that it likely maintained within physiological range, resulting in net hypercoaguability in response to an acute stressor (Austin & Patterson 2013, Lukas 2010, Thrall *et al.* 2007, von Känel *et al.* 2001a, Womack *et al.* 2003). However, other risk factors, including chronic stress, have been observed to modulate haemostasis, altering the balance between coagulation and fibrinolytic potential (Lukas 2010, von Känel *et al.*

2001a). The review of haemostasis represents a valuable tool for monitoring physiological and pathological processes, and may offer the advantage of detecting early stage DIC before the onset of excessive and uncontrollable microthrombus formation, and bleeding tendencies (Siroka *et al.* 2011, Monreal *et al.* 2000). While considerable information is presently available on coagulation and fibrinolytic potential in wild ruminants (Hussein *et al.* 2010, Peinado *et al.* 1999, Vengušt *et al.* 2002), several studies have concluded that extensive quantitative differences in coagulation properties exist among mammalian species (Gentry 2004, Hussein *et al.* 2010). The reference values obtained in this study address the need for species-specific data on haemostasis, and may be useful in the clinical and laboratory examination of impala.

In the present study, the minimal significant change in aPTT in the impala indicates that the intrinsic system of the coagulation pathway was not substantially altered in response to the chronic effects of confinement and repeated stress of immobilisation. Unchanged aPTT after stress is consistent with what was observed by von Känel *et al.* (2004), who suggested that it was too crude a measurement to mirror changes in the other, more sensitive components, of the coagulation cascade. Small sample size and high variation can perhaps also be attributed to the lack of significance observed in aPTT.

The more considerable significance observed in the PT of the impala in response to the effects of confinement and repeated immobilisation may be attributed to a more substantial alteration of the extrinsic coagulation pathway. Siroka *et al.* (2011) also reported an increased PT in fallow deer (*Dama dama*), which they attributed to the possible stress experienced by the animals. In addition, it may indicate that PT assays are more sensitive to show trends in coagulation potential. The PT increase also mirrors the FGM response and decline in body weight and condition. This suggests that secretion of factors important to the extrinsic pathway may have also been reduced.

The significantly reduced TT in the impala suggests considerably increased activity of the common coagulation pathway in response to the chronic effects of confinement and repeated stress of immobilisation. From these observations we suggest that as the trials progressed and the physiological condition of the animals worsened, there was a significantly decreased clotting time in response to metabolic and psychological stressors. The hypercoaguable state fostered by chronic stress observed in the present study is consistent with the supporting

literature (Austin *et al*. 2013, Lukas 2010, von Känel *et al.* 2003, von Känel *et al*. 2001a). Further, these results are in line with the observations of Dr. Neville Pitts and Dr. Jacobus Raath, who have observed an increased prevalence of DIC in stressed animals (personal communication). We observed that TT in the impala adapted to the chronic effects of confinement and repeated stressors, as did other parameters (including body weight and condition, FGM concentration, and ROS generating capability of whole blood) measured in the impala during the course of the current study. This is not consistent with the findings of von Känel *et al.* (2004) and others, who proposed that stress-induced procoagulant responses showed an inability to adapt or habituate to repeated stressors of the same type (Hamer *et al.* 2006, Lukas 2010, von Känel 2015).

A significant factor associated with the reduced TT result is the increase in fibrinogen concentration observed in the impala as the trials progressed. Only once fibrinogen began to return to the initial concentration did the TT increase back to its initial result. This observation can perhaps be attributed to the dual role of fibrinogen as a pivotal precursor in the coagulation cascade, and as an APP in the inflammatory response. In the present study, the increased fibrinogen levels observed in response to heightened APR activity is consistent with the supporting literature (Epstein & Brainard 2011, Factor 2007, Hantgan *et al.* 2001). The significant decline in fibrinogen concentration observed in the impala as the trials progressed may be attributed to the down-regulatory activity of anti-inflammatory stress hormones (Acevedo-Whitehouse & Duffs 2009).

For the purpose of this study we used thiafentanil oxalate (A3080) as the objective of the principle trial was to assess the optimal dose of thiafentanil for use in impala. Thereafter, naltrexone hydrochloride (naltrexone) was used to reverse the effects of immobilisation in the impala. The data obtained at immobilisation and then 30 min later represent the acute responses to immobilisation in this study. One potential limitation of the present study is that dose of the opioid was not considered in the model.

The significant reduction in cortisol concentration in response to 30 min of immobilisation in the impala may reflect the modulatory action of immobilising agents on the neuroendocrine response, and the down-regulating effect on GC secretion (Marana *et al.* 2010, Sakai *et al.* 1995).

The 30 min of immobilisation of the impala also increased the ROS generating capability of whole blood. This result is not consistent with the supporting literature, which reported a depression in bactericidal activity in response to other immobilising agents (Colucci *et al.* 2013, Heine *et al.* 2000, Lisowska *et al.* 2013). However, there are many examples showing that not all opioid drugs share the same immune profile, some seem to have no effects on immune function, whereas other opioids tend to be immunosuppressive or immunostimulatory. Further, duration of immobilisation and dosage of opioid appear to be mitigating factors to how individual opioids affect the immune system, with short term/low dose administration of immobilising agents seeming to have a positive impact on the immune system (Liang *et al*. 2016).

The significantly decreased activity of the common coagulation pathway observed in the impala in response to a 30 min immobilisation period indicates a significant effect of immobilisation on an individual's ability to form a clot. Immobilising agents have been reported to inhibit platelet function and decrease blood viscosity, thereby reducing the prevalence of thrombosis (Beilin *et al.* 2006, Binici *et al.* 2015, Faraday 2002, Odoom 1995, Reinhart 1995).

The significant increase in fibrinogen concentration observed in the impala in response to both the chronic effects of confinement and a 30 min immobilisation period can perhaps be attributed to the dual role of fibrinogen as a pivotal precursor in the coagulation cascade, and as an APP in the inflammatory response. In the present study, the increased fibrinogen concentration in response to heightened APR activity is consistent with the supporting literature (Epstein & Brainard 2011, Factor 2007, Hantgan *et al.* 2001).

A major limitation is the limited data available for the parameters that were necessary to measure in this study, let alone for free-ranging, relatively undisturbed and healthy impala. This limitation makes it difficult to understand and interpret the full range of responses that can be expected in response to the initial confinement and later adaptation phases apparently shown by these animals. Since we were unable to collect all of the necessary samples at capture, this study also relies on the assumption that the animals were undisturbed in the bomas prior to the commencement of the trials.

5 CONCLUSION

Current rates of biodiversity decline are unprecedented and to a large extent attributed to environmental challenge and anthropogenic influence. Given the scope and magnitude, conservation management interventions must maximise efficiency and efficacy. Effective wildlife research and management often requires the capture and handling of animals. However, the evaluation of immobilisation, capture, relocation and confinement effects on target animals is often disregarded, despite the high potential for significant stress. While some species habituate to human disturbance, others exhibit extensive psychological and physiological deficits, which can pose a challenge to animal husbandry and conservation as well as wild immunology.

In the present study we measured HPA activity, the ROS generating capability of whole blood, secretion of APP, and coagulation potential to investigate the physiological stress response elicited during capture, confinement and immobilisation in impala. In response to the chronic effects of confinement and the repeated stress of immobilisation we observed an initial increase in HPA activity and common coagulation pathway activity, and resultant depletion in body reserves. There was a decline in HPA activity by T4, and corresponding immunesuppression. Thereafter, as animals habituated to stressors, there was a restoration in body weight and condition, and subsequent recovery of HPA activity to initial results. In response to 30 min of immobilisation the impala showed decreased HPA activity and coagulability, and immune-enhancement.

The present study attempted to use new techniques, such as LCC and secretion of APP, to progress the understanding of short- and long-term impacts of anthropogenic disturbances on wildlife fitness, and improve animal welfare during conservation interventions. As a discipline, it is apparent that physiology may be undervalued, possibly due to the invasive nature of some physiological measurement techniques (e.g. sampling of body fluids). We propose that the utilisation of physiological indicators, in addition to animal health indices, is essential to indicate patterns of stress and coping in free-living vertebrates. We caution that there are limitations to this approach and that a considerable about of fundamental research remains to be done to understand their potential role when diagnosing disease, determining prognosis, and monitoring the health status of these animals. We urge more critical and thorough assessments and interpretations of measures of physiological stress to be undertaken

hence, particularly, in regards to physiological baseline data of organisms in their natural environment. Further studies should be undertaken to expand our knowledge of individual differences such as temperate, age and physiological status. Variation due to diurnal and seasonal changes should also be taken into consideration.

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