The haematology of bobtail lizards (*Tiliqua rugosa*) in Western Australia: reference intervals, blood cell morphology, cytochemistry and ultrastructure

This thesis is presented for the degree of Research Masters with Training at Murdoch University.

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April 2014
DECLARATION

I declare this is my own account of my research and contains as its main content, work which has not been previously submitted for a degree at any tertiary educational institution.

Cheryl Ann Moller

April 2014
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I hope this thesis is a resource for those with an interest in reptile clinical pathology, who, like me, are disappointed with the lack of published information about our favourite scaly creatures.

Cheryl Ann Moller, budding reptile clinical pathologist and lizard lady for life, April 2014
Bobtail lizards (*Tiliqua rugosa*) are native to Western Australia. Haematological evaluation is useful for health assessment: the only previous study of the haematology of this species sampled just six lizards (Canfield and Shea, 1988). The main aim of this study was to produce reference intervals for bobtail haematology.

Over the summers of 2011/12 and 2012/13, heparinised venous blood was collected from 46 clinically healthy, captive adult bobtails in Perth. Complete blood counts and blood smear evaluations were performed. Cytochemical stains, transmission electron microscopy, and bone marrow cytology and histology facilitated further characterisation of the blood cells. Reference intervals with 90% confidence intervals were determined using Reference Value Advisor freeware (Geffré et al., 2011). The packed cell volume (PCV) was 0.10-0.44L/L (n=40). Total plasma protein by refractometry was 36-74g/L (n=39). Haemoglobin was 20-154g/L (n=32). The manual red and white blood cell counts were 0.28-1.03x10^{12}/L (n=38) and 2.75-30.76 x10^{9}/L (n=39), respectively.

Blood cell morphology was similar to that of other lizards - except the eosinophils which were uniformly vacuolated. A 200 cell leukocyte differential count was performed on each smear (n=46). Heterophils predominated (27-88%), with fewer lymphocytes (0-34%) and monocytes (1-27%), occasional eosinophils (0-22%) and basophils (0-20%). Thrombocytes were frequently clumped or present as bare nuclei. Slight polychromasia (0-7%) was typically present (n=45).

Many reference intervals were wide, particularly PCV, haemoglobin and white blood cell count. This was not unexpected as reptile haematology is influenced by many preanalytical factors.

Smears from 13 bobtails contained haemogregarine parasites, identified as probable *Hemolivia* species. There was evidence that this infection caused mild erythrocyte pathology.

The reference intervals were applied to the haematology of seven bobtails hospitalised with upper respiratory tract disease. Six bobtails possessed haematological evidence of inflammation. Thus the reference intervals appear to be clinically useful for the haematological assessment of captive bobtail lizards.
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ABBREVIATIONS

APTT- activated partial thromboplastin time
CLSI-IFCC - Clinical and Laboratory Standards Institute and International Federation of Clinical Chemistry
CV- coefficient of variation
EDTA- ethylenediaminetetraacetic acid
H&E- haematoxylin and eosin
MCH- mean corpuscular haemoglobin
MCHC- mean corpuscular haemoglobin concentration
MCV- mean corpuscular volume
N:C ratio- nuclear to cytoplasmic ratio
PAS- periodic acid-Schiff
PCV- packed cell volume
PT- prothrombin time
QALS- Quality Assurance and Laboratory Standards
RBC- red blood cell
SD- standard deviation
T. rugosa- Tiliqua rugosa
TP- total protein
URTD- upper respiratory tract disease
WBC- white blood cell
INTRODUCTION

*Tiliqua rugosa* is a common native Australian reptile known as the bobtail, shingleback or sleepy lizard (Figure 1). *Tiliqua rugosa*, also known as *Trachydosaurus rugosus*, (from the Latin *ruga* meaning wrinkled) was first described by J.E. Gray in 1825. Bobtails are classified in the order Squamata, meaning they possess scales, suborder Sauria (also known as Lacertilia; lizards) and family Scincidae (skinks).

![Figure 1: An adult bobtail lizard.](image)

*Tiliqua rugosa* is found Australia-wide, south of the Tropic of Capricorn (Figure 2). The species has been classified into subspecies according to tail length, colour distribution and head scale pattern. The most common subspecies in mainland Western Australia is *Tiliqua rugosa* subspecies *rugosa*. Other subspecies found in Western Australia are *Tiliqua rugosa* subspecies *konowi* which is found exclusively on Rottnest Island, and *T. rugosa* subspecies *palarra* which is found in Shark Bay. *T. rugosa* subspecies *aspera* is the subspecies found in eastern Australia.
The lifespan of bobtails has been reported as 20 years or more. They reach sexual maturity between 3 and 5 years of age (Bull, 1995). Bobtail lizards stay within a specific home range for most of their life (Bull and Burzacott, 2006). They are monogamous, and in spring they partner with their mate and breed in November. Gestation is 6 to 7 months (Munns and Daniels, 2007). Bobtails are viviparous: meaning they bear live, precocious young, and they produce one to four young each pregnancy. Viviparity is thought to be an evolutionary adaptation to the southern distribution of the species, where egg incubation is inhibited by low temperatures (Bush et al., 1995; Sites Jnr et al., 2011).

*Tiliqua rugosa* is a large skink with an adult snout to vent length (SVL) greater than 280mm (Bull, 1995). They exhibit a low degree of sexual dimorphism. Females tend to have a wider and shorter tail, as well as a wider body to accommodate the young which develop in the lateral aspects of the coelomic cavity. Identification of males can be achieved by manual eversion of the hemipenes. Eversion of hemipenes can be performed by applying pressure on the ventral tail base, but this can be difficult (Cogger, 1996).
Bobtails are diurnal and omnivorous but primarily herbivorous, storing excess energy as fat in the subcutaneous tissues of the tail and coelomic fat bodies (Dubas and Bull, 1991). The preferred body temperature of *T. rugosa* is 34°C. Bobtails brumate in colder weather: they enter a hibernation-like state where physiological processes are down-regulated (Licht, 1965).

Bobtails are seen frequently in the Perth metropolitan area, especially between late spring and early autumn (Haight, 2013). They are the native reptile most frequently admitted to veterinary clinics and wildlife hospitals. In sick or injured domestic animals, assessment of haematology and clinical chemistry is frequently performed to assess the severity/extent of disease, and monitor progress. These analyses are less frequently performed in wildlife due to the expense and lack of published “normal” reference intervals. In non-mammalian species including reptiles, this becomes particularly important as “normal values” are much more variable (Campbell, 2006).

In addition to trauma, the other main reason for admission of wild bobtails to wildlife hospitals is a condition colloquially known as “URTI” (upper respiratory tract infection) or “bobtail flu” (Haight, 2013). Little is known about this disease as it has not been well studied to date. An infectious agent is presumed responsible, but not proven, so henceforth it will be referred to as upper respiratory tract disease (URTD).

Typically, there are few abnormalities on the complete blood count of animals with upper respiratory tract disease. One of the most well-studied upper respiratory tract diseases of reptiles is *Mycoplasma agassizii* infection in tortoises. The main haematological finding in affected tortoises is a mild to moderate decrease in haemoglobin concentration, assumed to be due to inflammation (Jacobson et al., 1991).

During microbial infections, the immune response and the subsequent effect on haematology can differ depending on the type of organism responsible. For example, increased numbers of eosinophils may be seen with nematode infections. A left shift and toxic changes in the neutrophils or heterophils (the reptilian equivalent of the mammalian neutrophil) may be seen in bacterial infections (Strik et al., 2007). It is not known whether...
there are any such changes in the haematology of bobtails with upper respiratory tract disease, and whether these changes may elucidate anything further about the aetiology.

Examination of bobtails with upper respiratory tract disease typically reveals mucous membrane pallor and a dull demeanour - suggestive of a systemic response to disease. Thus haematologic abnormalities may be detectable.
AIMS

There were three aims of this study. The first aim was to characterise the blood cells of the bobtail lizard (*Tiliqua rugosa*) using light microscopy, cytochemistry and transmission electron microscopy. The second aim was to formulate reference intervals for the haematology of healthy captive bobtail lizards. The third aim was to use the reference intervals to evaluate the haematology from a cohort of bobtails with upper respiratory tract disease, determine whether there were any significant differences, and thus whether haematology is useful in diagnosis of the disease, or may further elucidate the aetiology of the disease.

The first and second aims are addressed in Chapter 1, where the focus is on the haematology of healthy bobtail lizards. Chapter 2 utilises the reference intervals generated in Chapter 1 to evaluate the haematology from bobtails with upper respiratory tract disease, thereby achieving the third aim. In addition, several opportunities arose during this study to further investigate pathological conditions of bobtail lizards, and these are also included in Chapter 2. The additional pathological findings broaden the scope of this thesis to investigate haemogregarine parasite infections in ticks and bobtail tissues using histopathology and tick gut smears, to review histopathology cases from bobtails with upper respiratory tract disease, and finally, to examine post mortem bone marrow cytology and histopathology from a bobtail with skull fractures and myiasis.
**LITERATURE REVIEW**

**Haematology of reptiles**

The clinical assessment of illness in reptiles can be difficult, so laboratory evaluation can provide valuable information (Hernandez-Divers et al., 2004). Haematology and biochemistry profiles have been described as useful screening tools to detect disease in reptiles, and for monitoring response to treatment (Campbell, 2006; Johnson and Benson, 1996).

**Blood collection**

The blood volume of reptiles is approximately 5-8% of body weight. Of that, 10% may be safely collected. The recommended site of blood collection varies with the family and the size of the reptile but in larger lizards, the ventral coccygeal (tail) vein is the most common site used (Strik et al., 2007). The ventral coccygeal vein runs along the midline of the ventral aspect of the coccygeal vertebrae, with parallel paired lymphatic vessels (Ottaviani and Tazzi, 1977). To avoid the hemipenes of male lizards, Hernandez-Divers et al. (2004) recommends that blood be collected from the middle of the tail, with the optimal site between 25 and 75% of the distance of the tail. A ventral or lateral approach may be used. For the ventral approach, the site should be disinfected with iodine or alcohol and a 22-25G, 5/8 to 1 inch (16-25mm) needle inserted between the scales of the midline, then once through the skin, directed at an angle of 60-90° until just below the ventral aspect of the coccygeal vertebrae. Slight negative pressure is applied while the needle is gently redirected until blood flows into the syringe (Hernandez-Divers and Garner, 2003; McCracken, 1994). Pre-heparinising the needle and/or syringe prior to venepuncture may also assist the prevention of clot formation, and is particularly useful for slow blood collections (Strik et al., 2007).
As lymphatics run parallel to the ventral coccygeal vein, accidental puncture of lymphatics during collection can occur (Gottdenker and Jacobson, 1995). This may be seen as filling of the syringe with clear fluid. If this occurs, the sample should be discarded and a new site attempted.

In bobtail lizards, blood can be collected from the ventral coccygeal vein or the ventricle (Canfield and Shea, 1988). Ventricle puncture is invasive and sedation or general anaesthesia is recommended to minimise the risk of excessive trauma or laceration of the heart (Hernandez-Divers et al., 2004). Blood collection from the ventral coccygeal vein is difficult in this species for two reasons. The first reason is that there is usually a large amount of stored fat in the tail of healthy lizards, making access to the vein problematic (Simone Vitali, pers. comm.) (Figure 3). Secondly, the ventral coccygeal vein and lymphatics traverse through the V-shaped ventral processes on the coccygeal vertebrae (the chevron bone) (Davies, 1981; McCracken, 1994). These ventral processes may be encountered during venepuncture, necessitating redirection (McCracken, 1994) (Figure 4).

To prevent coagulation, reptile blood can be transferred into potassium ethylenediaminetetraacetic acid (EDTA) or heparin anticoagulant for haematological analysis (Campbell and Ellis, 2007).
Figure 3: Cross section of the tail of *T. rugosa*, post mortem. Note the large aggregates of fat, the central coccygeal vertebra, and the ventral coccygeal vein (arrow). Reproduced with kind permission from Dr. Julia Galvez.

Figure 4: The cranial aspect of coccygeal vertebra 5 from an adult bobtail, showing the large ventral process of the coccygeal vertebrae (white arrow). The white asterisk (*) indicates the location of the ventral coccygeal vein. Image credit Gary Allen.
Haematologic analysis

Following collection of reptile blood, rapid analysis (within 24 hours) is recommended as cells quickly deteriorate (Vap et al., 2012). The analysis of reptilian haematology can be accomplished in a similar way to that of mammals. Like fish, amphibians and birds, reptiles have nucleated erythrocytes and nucleated platelets known as thrombocytes. When automated haematology analysers process a mammalian blood sample, chemicals lyse the anucleate blood cells in order to count the leukocytes. In non-mammalian species, the persistence of erythrocyte and thrombocyte nuclei following lysis usually precludes accurate automated counts (Russo et al., 1986). Analysers that are capable of evaluating non-mammalian haematology must be validated for this use (Vap et al., 2012). Validated analysers may not be widely available, thus manual methods are largely used for the haematology of reptiles and other non-mammalian species. Manual methods are less precise than automated methods. Coefficients of variation (CV) for manual methods have been reported between 13% and 40%; this is compared to automated methods, which should have a CV of less than 5-10% (Jensen and Kjelgaard-Hansen, 2010; Russo et al., 1986; Vap et al., 2012).

Red blood cell counts, white blood cell counts and thrombocyte counts are usually derived manually using a haemacytometer counting chamber. An aliquot of blood is diluted with a solution: in reptiles, most often the Unopette® dilution system or Natt & Herrick’s stain solution (Natt and Herrick, 1952). Then the cells are counted and corrected for the dilution factor to produce the cell count (Campbell and Ellis, 2007). Unopette® production has been discontinued but other similar products are available. A phloxine stain may also be used with or without the Unopette® (or similar) dilution method which stains granulocytes orange-pink (Dein et al., 1994). Natt & Herrick’s solution is advantageous as it is also isosmotic and thus preserves cell morphology, and it is also a stain, staining all cells variable intensities of purple with methyl violet. This allows ready differentiation of leukocytes from erythrocytes (Natt and Herrick, 1952). Errors can occur with the Natt & Herrick’s method if there is improper dilution of blood, e.g., due to faulty pipettes, poor mixing, or inadequate filling of the counting chamber. For this reason, it is recommended to compare the white
blood cell count to an estimate from a blood smear, and to compare the red blood cell count to the packed cell volume (PCV) (Stacy et al., 2011).

As in mammals, microhaematocrit centrifugation is the most practical and reproducible method of obtaining the PCV for reptile blood (Campbell, 2006; Rizzi et al., 2010).

Haemoglobin is measured using a haemoglobinometer such as a Coulter counter or a haematology analyser using cyanmethaemoglobin (or comparable) methods. Cyanmethaemoglobin-type methods are similar to those performed in mammals, however non-mammalian blood must typically be centrifuged prior to measurement of haemoglobin as the persistence of erythrocyte nuclei following lysis will interfere with results (Campbell and Ellis, 2007).

Blood smears must also be examined. Smears should be made directly following blood collection and may be prepared as for mammals; some authors advocate the use of two coverslips (“the coverslip method”) rather than two glass microscope slides to reduce mechanical rupture of the cells (Strik et al., 2007). White blood cell count estimates, leukocyte differential counts, and assessment of morphology are performed on smears just as in mammals (Stacy et al., 2011).

Before considering the unique characteristics of reptile and specifically, lizard haematology, it is important to consider the preanalytical factors which influence the results.

**Preanalytical factors influencing reptile haematology**

Compared to mammals, the results from reptile haematological evaluation are influenced by a wider range of both intrinsic and extrinsic preanalytical factors (Campbell and Ellis, 2007). These factors must be considered when interpreting results.
Intrinsic (biological) factors

Species differences

Within the class Reptilia, there is a wide variation in the morphology and differential counts of leukocytes. There may also be differences in the differential leukocyte counts even within a genus, as has been reported in the giant lizard (Martinez-Silvestre et al., 2005).

The size and volume of erythrocytes varies from a mean corpuscular volume (MCV) of less than 300fl in lizards, to between 300 and 500fl in snakes, to greater than 500fl in chelonians (turtles and tortoises) (Campbell, 2006). Lizards have the highest red blood cell counts (RBC; 1-1.5 x 10^{12}/L), snakes are intermediate (0.7-1.6 x 10^{12}/L), and chelonians have the lowest (<0.5 x 10^{12}/L), suggesting an inverse relationship between red cell size and circulating numbers (Campbell, 2006; Sypek and Borysenko, 1988).

Leukocyte differential counts can vary widely. For example, turtles can have up to 20% circulating eosinophils, whereas in some reptiles such as the eastern diamondback rattlesnake, eosinophils have not been identified at all (Alleman et al., 1999; Campbell, 2004).

Leukocyte morphology can also be quite variable in reptiles. In some reptiles such as the Gila monster, the heterophils have an oval nucleus, whereas in others such as the iguana, heterophils have a lobulated nucleus (Cooper-Bailey et al., 2011; Harr et al., 2001).

Sex and reproductive status

The sex of the reptile can also influence results, with males of many reptile species possessing a higher PCV and haemoglobin concentration. In other reptiles such as iguanas, PCV and haemoglobin are increased in females (Anderson et al., 1997; Harr et al., 2001; Strik
et al., 2007; Wojtaszek, 1991). Lower circulating monocyte numbers have also been reported in females of some reptile species such as the yellow-headed temple turtle (Chansue et al., 2011). These differences may be due to the effect of sex hormones or reproductive activity (Jacobson and Origgi, 2002).

**Other intrinsic factors**

The effects of age are similar in reptiles and mammals (Harr et al., 2001). Juvenile reptiles often have higher lymphocyte counts than adults (Pienaar, 1962). Nutritional status may cause a direct effect on parameters, such as lower erythroid values due to iron deficiency. Poor nutrition, and captive living in some species, can cause a corticosteroid stress response which results in a decrease in circulating lymphocytes and increase in heterophils (Campbell and Ellis, 2007; Cartledge et al., 2005; Morici et al., 1997; Salakij et al., 2002).

**Extrinsic (non-biological) factors**

Extrinsic factors pertaining to the evaluation of reptile haematology must also be considered.

**Temperature and season**

An important difference in the physiology of mammals and reptiles is the type of metabolism. Mammals and birds are endothermic, so body temperature is maintained within a narrow range by the complex interactions of cutaneous thermosensors, hormonal, autonomic and central neurological pathways (Bicego et al., 2007). These interactions expend considerable energy. All metabolic reactions in the body operate at optimal levels within this narrow temperature range.

Reptiles are ectothermic, so body temperature is determined by the temperature of the surroundings. This has the benefit of conserving energy. However, just as in mammals, all
metabolic reactions in reptiles have a temperature at which their function is optimal, known as the preferred body temperature (34°C in bobtails). Above or below this temperature, reactions are less efficient. Reptiles attempt to optimise metabolism by maintaining their body at the preferred temperature by the regulation of cutaneous blood flow and heart rate, and behavioural means such as moving in and out of heat, opening their mouth or panting. The precise mechanisms of regulation are not fully understood but may be similar to those in mammals (Bicego et al., 2007).

Therefore, in reptiles, the metabolic rate is dictated by the ambient temperature. In non-captive reptiles in particular, this means that season has a strong influence on physiology, regulated by the circadian rhythm. The circadian rhythm involves interactions between the pineal gland (particularly the hormone melatonin), the eye, hypothalamus, thyroid and adrenal glands (Seebacher and Franklin, 2005). In winter, where temperatures are too low for metabolic processes to occur efficiently, reptiles will enter a state of brumation. This downregulation is sometimes referred to as hibernation however hibernation is not considered an appropriate term for reptiles as they do not actually “sleep” during this time (Blood and Studdert, 1999; Rial et al., 2010).

During brumation, all metabolic processes are downregulated including the production of blood cells and the maintenance of immune surveillance. Circulating numbers of erythrocytes and heterophils decrease (Campbell, 2006; Montali, 1988). The thymus and splenic white pulp involute during winter, most likely as a result of an increase in endogenous corticosteroids, causing a drastic decrease in lymphocyte numbers due to apoptosis (Campbell, 2006; El Ridi et al., 1988; Kruman, 1992). This results in a decrease in circulating lymphocytes (lymphopenia). Circulating lymphocyte numbers increase again once the reptile resumes normal activity in spring and lymphoid tissues are re-populated with lymphocytes (El Ridi et al., 1988).
Hydration status and reproductive activity may also vary seasonally. Dehydration in the dry season or the summer may cause elevations in erythroid values and plasma and serum proteins (Harr et al., 2001).

Thus the season and ambient temperature strongly influence the physiology and haematology of reptiles.

**Venepuncture site**

The venepuncture site may also cause variations in haematologic data. In reptiles, as lymphatic vessels run in parallel with blood vessels, it is common for blood samples to be diluted with lymph (McCracken, 1994). There are no lymphatics parallel to the jugular vein in some species, or the ventricle of the heart (Campbell and Ellis, 2007). Lymph contamination of a sample falsely elevates the lymphocyte count, as well as resulting in haemodilution which decreases cell counts and erythroid values (Gottdenker and Jacobson, 1995).

**Anticoagulant**

The type of anticoagulant into which blood is collected will also affect the results of reptile haematology. EDTA anticoagulant causes lysis of red blood cells in some reptiles, particularly turtles and tortoises (Strik et al., 2007; Sykes and Klapake, 2008). Using lithium heparin anticoagulant is advantageous as it enables haematology and biochemistry analyses to be performed from the same sample. However, it is well recognised that lithium heparin causes clumping of reptile thrombocytes, precluding accurate thrombocyte counts (Strik et al., 2007). Lithium heparin also imparts a faint blue tinge to the plasma protein in the background of the blood smear and may result in paler staining of cells with Romanowsky stains (Hawkey and Dennett, 1989; Strik et al., 2007).
Staining of blood smears

The type of stain used on blood smears can also affect results. Rapid stains may cause damage to or under-staining of cells (Sykes and Klaphake, 2008). Basophil granules may dissolve with aqueous stains such as Giemsa, thus the use of alcohol-based Romanowsky stains is recommended (Campbell, 2006; Raskin, 2000).

Thus there are many intrinsic and extrinsic preanalytical factors which can influence the haematology of reptiles. These factors must be known and controlled where possible for meaningful evaluation of haematology results.

Characteristics of reptile blood cells

There are considerable differences in the physiology of reptiles and mammals. In order to effectively analyse and interpret haematological data from reptiles, it is important to review the haematopoiesis (production), morphology, function, physiology and common pathology of the peripheral blood cells of reptiles. There are few published studies on the haematology of reptiles, so, in this review, where indicated, some extrapolations have been made from avian studies.

Haematopoiesis

In all vertebrates, the initial sites of haematopoiesis in the embryo are the blood islands of the yolk sac: aggregates of mesoderm-derived haemangioblasts surrounded by supporting endoderm (Baumann and Dragon, 2005). The haemangioblasts themselves originate from the area opaca (Zon, 1995). In the blood islands, the inner haemangioblasts differentiate primarily into erythrocyte precursors and some thrombocytes and monocytes, and the outer haemangioblasts eventually form endothelial cells (Baumann and Dragon, 2005; Zon, 1995).
The yolk sac produces the haematopoietic cells until the bone marrow is established. The bone marrow is derived from embryonic mesoderm cell clusters present in the mesentery and the lining of blood vessels, known as the aortic-gonad-mesonephros (AGM) region (Baumann and Dragon, 2005; Cavlac, 2009; Vasse and Beaupain, 1981). Differentiation of precursor cells to the erythroid or myeloid cell lines is influenced by the key transcription factors GATA1 (favours erythroid) and PU.1 (favours myeloid) in all vertebrates (Morera and MacKenzie, 2011).

The onset of bone marrow activation is highly variable between reptile species. In the tortoise, it occurs just prior to hatching (Garner et al., 1996; Vasse and Beaupain, 1981). Post-natal haematopoiesis occurs primarily in the sinuses of the bone marrow; the liver and spleen provide a minor contribution in the neonate (Baumann and Dragon, 2005; Claver and Quaglia, 2009; Garner et al., 1996; Keymer, 1981; Sano-Martins et al., 2002). Foci of extramedullary haematopoiesis are retained in the liver and spleen of some adult reptiles (Meints et al., 1975).

There are seven types of blood cells normally found in the peripheral blood of post-natal reptiles: erythrocytes, heterophils, eosinophils, basophils, monocytes (including azurophilcs), lymphocytes and thrombocytes.

**Erythrocytes**

**Erythropoiesis**

Primitive (pre-natal) and definitive (post-natal) reptilian erythrocytes are produced by the yolk sac but originate from different progenitor cells and contain different haemoglobin molecules (Baumann and Dragon, 2005; Vasse and Beaupain, 1981; Zon, 1995). Most primitive erythrocytes progressively undergo apoptosis by birth/hatching but some appear to persist for the first few years of life (Vasse and Beaupain, 1981; Zon, 1995).
Reptilian bone marrow contains only very few (<6%) erythrocyte precursors (Glomski and Pica, 2011). The maturation sequence of the erythroid series in reptiles is very similar to that seen in birds with a progressive increase in cell size, decrease in nuclear to cytoplasmic ratio and increase in eosinophilia of the cytoplasm due to progressive haemoglobinisation. In order of maturation, from least mature, the erythroid series is composed of rubriblasts, prorubricytes, basophilic rubricytes, early polychromatic rubricytes, late polychromatic rubricytes, polychromatic erythrocytes and mature erythrocytes (Campbell, 2004). In reptiles, basophilic rubricytes are the first erythroid stage normally released into the peripheral blood from the bone marrow (Campbell and Ellis, 2007). In a study of erythropoiesis in the common Australian garden skink and the American chameleon lizard, which utilised the metabolic incorporation of H3-thymidine radioactive nucleosides into DNA, it took approximately 14 days for rubriblasts to generate polychromatophilic erythrocytes (Alibardi, 1994).

The lifespan of reptilian erythrocytes is long and can vary depending on the ambient temperature. Erythrocyte lifespan was reported as 600-800 days in the box turtle (by C14 methyl-labelled glycine radioisotope) and 180-300 days in the American alligator (by P32 or tritium labelled diisopropylfluorophosphosphate). This is compared to only 28-45 days in birds (by Na2Cr51O4 radioisotope) and 110-120 days in the dog (by Cr51) (Brace and Altland, 1955; Cline and Waldman, 1962; Rodnan et al., 1957; Vacha, 1983). The long lifespan is likely related to the low basal metabolic rate of reptiles (Campbell, 2006; Rodnan et al., 1957). Aged red blood cells are removed by splenic macrophages as in mammals (Strik et al., 2007).

**Morphology**

The mature erythrocytes of reptiles are nucleated. In mammals, the evolutionary loss of the inactive nucleus of the metarubricyte enabled an increase in haemoglobin concentration and therefore oxygen-carrying capacity of the mature erythrocyte (Ji et al., 2008).
Reptilian erythrocytes are large, 15-20µm in diameter, oval to elliptoid cells, with an orange-pink cytoplasm on Romanowsky-stained smears, a central oval nucleus with dense purple chromatin and irregular nuclear margins (Campbell, 2006; Glomski and Pica, 2011). The shape of the erythrocyte is the result of an intricate network of filaments which form the membrane skeleton: interconnected actin and spectrin filaments, and vimentin and synemin proteins which attach to the nucleus (Desser and Weller, 1979; Glomski and Pica, 2011; Kingsley et al., 2004). The alteration in shape from the round polychromatophilic erythrocyte to the ovoid mature erythrocyte is due to the development of microtubules which form the marginal bands in the mature erythrocyte (Glomski and Pica, 2011).

Ultrastructural examination of reptile erythrocytes with transmission electron microscopy reveals ovoid to elliptical cells with a uniformly electron-dense cytoplasm, and a nucleus with peripheral heterochromatin (Casal et al., 2007; Martinez-Silvestre et al., 2005). Intracytoplasmic inclusions ultrastructurally suggestive of degenerate mitochondria or other organelles have been seen in erythrocytes from the desert tortoise and Hawaiian green turtle (Alleman et al., 1992; Work et al., 1998).

Immature erythrocytes or polychromatophils may be found in the circulation of healthy reptiles (Campbell, 2006). They are round cells with a large round nucleus and a characteristic “checkerboard pattern” to the chromatin. Polychromatophils are smaller than mature erythrocytes, with a lower MCV, higher nuclear to cytoplasmic ratio, and a more basophilic cytoplasm due to incomplete haemoglobinisation (Campbell, 2006; Strik et al., 2007).

**Function**

The primary role of erythrocytes is the exchange of oxygen and carbon dioxide between the lungs and tissues. As in almost all other vertebrates, the transport of oxygen is by binding to the iron-containing haeme molecule in haemoglobin within the cytoplasm of the red blood
cell. Reptiles have several types of haemoglobin molecules including haemoglobin A and D. Variability in the oxygen affinity of individual erythrocytes is thought to be due to variations in erythrocyte age (Frische et al., 2001; Weber et al., 2004). The structure of the haemoglobin molecules are well conserved across reptile species, however there are differences in oxygen affinity between orders, with lizard haemoglobin having greater affinity and therefore less oxygen unloading than chelonians over a range of ambient temperatures (Coates, 1975; Johansen et al., 1980; Strik et al., 2007; Torsoni and Ogo, 1995). Temperature also affects oxygen affinity, with reptiles from environments with narrower temperature ranges possessing higher oxygen affinities (Stawski et al., 2006).

Energy is required by red blood cells for many functions including the maintenance and alteration of shape (i.e., deformability by microfilament rearrangement), maintenance of ion gradients, cellular repair, and the manufacture of haemoglobin (Bonora et al., 2012; Speckner et al., 1989). Energy in the form of ATP is produced by nucleated red blood cells via aerobic metabolism of carbohydrates in the tricarboxylic acid cycle, as well as a lesser component via the metabolism of amino acids (Mauro and Isaaks, 1997; Phillips et al., 2000). In contrast, erythrocyte metabolism in mammals is purely anaerobic as most organelles, including mitochondria, are extruded from the cell prior to entering circulation in order to maximise cytosolic space for haemoglobin (Phillips et al., 2000).

Erythrocytes also play a minor role in innate immunity. Peptide fragments of reptile haemoglobin have been shown to possess antimicrobial activity against fungi, as well as Gram positive and Gram negative bacteria (Morera and MacKenzie, 2011; Parish et al., 2001).

**Laboratory evaluation**

Compared to mammals and birds, reptiles have a lower red blood cell count, haemoglobin concentration, and PCV, resulting in a lower oxygen-carrying capacity (Strik et al., 2007). The
reptile red blood cell count is highest just before brumation, and lowest afterwards when they resume normal metabolic activity (Campbell, 2004). The PCV of healthy reptiles ranges between 0.20 and 0.40L/L, with a typical red blood cell count of 1 to $1.5 \times 10^{12}$/L and haemoglobin concentration between 60 and 100g/L (Campbell, 2006).

On blood smears, small clear inclusions may be seen within the red blood cell cytoplasm which are considered artefacts of smear preparation (Reagan et al., 2008). Small basophilic cytoplasmic inclusions may be seen which represent degenerative organelles and may be confused with viral inclusion bodies or haemoparasites (Alleman et al., 1992; Campbell, 2006). Haemoglobin crystals are also occasionally identified in the red cell cytoplasm and are not considered clinically significant (Strik et al., 2007). Rarely, anucleate erythrocytes, known as erythroplastids, may be seen in reptilian blood (Glomski and Pica, 2011). These may be due to physiological enucleation, or they may be artefacts of smear preparation (Fudge, 2000; Glomski and Pica, 2011).

Polychromatophils are normally present on blood smears of healthy reptiles, usually less than 1 per 100 mature red cells (Campbell, 2006). Reticulocytes are also usually present and are identified using a supravital stain such as new methylene blue, as for mammalian blood. Aggregate (more cytoplasmic RNA) and punctate (less RNA) reticulocytes may be identified, with the blue-staining RNA encircling the nucleus (Campbell, 2006; Salakij et al., 2002). Healthy reptiles usually have less than 1.5 to 2.5% circulating reticulocytes. Quantification of polychromasia is considered the best way of interpreting red cell regeneration in reptiles (Hawkey and Dennett, 1989).

Mild anisocytosis and an occasional poikilocyte are considered normal findings in healthy reptiles (Campbell, 2006; Hawkey and Dennett, 1989). Occasional mitotic figures in erythrocytes may also be seen (Campbell, 2006).
**Abnormalities**

Polychromatophil numbers physiologically increase following brumation as the metabolism resumes normal activity. They may also be increased in young reptiles or those undergoing ecdysis (Campbell, 2006).

More than an occasional poikilocyte is considered significant in reptiles (Hawkey and Dennett, 1989).

An increase in the red blood cell count or packed cell volume of reptiles is most often due to haemoconcentration resulting from dehydration (Rossini et al., 2011).

As in mammals, anaemia in reptiles may be regenerative or non-regenerative. Note that lymph dilution may falsely reduce the primary erythroid values.

Regenerative anaemia can be caused by haemorrhage or haemolysis. Haemorrhage may be due to trauma, phlebotomy, or ectoparasite infestation. If the haemorrhage is external, it can cause iron deficiency which may cause hypochromasia on the blood film. Haemolysis can result from toxins or haemoparasites (see below). The reptilian regenerative response is slower than that of mammals, with 4 weeks before significant polychromasia is observed, and up to 8 weeks for a maximal response (Sheeler and Barber, 1965; Strik et al., 2007). Because of the smaller size of polychromatophils compared to mature erythrocytes, increased anisocytosis is expected to correlate with the regenerative response. Red cell mitoses may also be increased with regenerative anaemia (Campbell, 2006). Basophilic stippling is usually related to red cell regeneration but may also be seen with iron deficiency or lead toxicity (Campbell, 2006; Reagan et al., 2008).

Regenerative anaemia with poikilocytosis and an increase in leukocyte numbers was reported in a small cohort of seven bobtail lizards which had green-coloured plasma due to
hyperbiliverdinaemia. The proposed pathogenesis was haemolysis due to a contagious pathogen, but no definitive diagnosis was made (Pennacchio et al., 2003).

Non-regenerative anaemia may be due to decreased erythropoiesis secondary to inflammatory disease, chronic renal or hepatic disease, or hypothyroidism (Campbell, 2006; Campbell and Ellis, 2007).

*Haemogregarines in reptiles*

Haemogregarine intraerythrocytic protozoan parasites have been frequently reported in reptiles and are usually an incidental finding, seldom causing disease (Campbell, 2004). Usually less than 1% of erythrocytes are infected, but infection rates of up to 64% have been reported, with no apparent clinical consequences (Brown et al., 2006). Haemogregarine genera cannot be distinguished by their gamont morphology alone, but those commonly found in reptiles include *Hemogregaria*, *Hemolivia*, and *Hepatozoon*. On Romanowsky-stained smears, the gamonts appear as ovoid to sausage-shaped, large pale basophilic inclusions with a central or eccentric dark nucleus. Normally only one parasite will be present in the cytoplasm of the red cell, rarely two (Strik et al., 2007). When infected erythrocytes are examined using transmission electron microscopy, gamonts are found within a parasitophorous vacuole and they typically possess a nucleus, dense granules, vacuoles and micronemes (Salakij et al., 2002). Micronemes are organelles found in the apical complex of protozoa in the phylum Apicomplexa. Other organelles also found in the apical complex including the polar ring, conoid, rhoptries, dense granules and microtubules. Micronemes have an important role in host cell invasion (Tomley and Soldati, 2001).

The lifecycle of haemogregarines involves sexual reproduction or sporogony in a biting invertebrate host with transmission of sporozoites during the biting process or following ingestion of the invertebrate. The asexual reproduction or merogony phase occurs in the reptile host (Keymer, 1981). Vertical transmission has also been reported (Telford Jr., 1984).
Other parasites that can infect reptile erythrocytes include coccidia such as *Schellackia* and *Lainsonia* (which can also infect leukocytes), *Plasmodium* species, *Sauroleishmania* and various piroplasms (Strik et al., 2007).

**Haemogregarines in bobtail lizards**

*Amblyomma limbatum* and *Aponomma hydrosauri* are two tick species that parasitise bobtail lizards in south-western Australia. These two tick species can carry the parasite *Hemolivia mariae* (Sharrad and King, 1981; Smallridge and Paperna, 1997b). *Hemolivia mariae* is a haemogregarine protozoan that infects the erythrocytes of bobtails. *Hemolivia mariae* is in the class *Coccidia*, suborder *Adeloreorina*, family *Haemogregarinidae* (NCBI, 2013; Smallridge and Paperna, 1997a; Smallridge, 1998).

In most bobtails, infected red blood cells make up less than 1% of the circulating red cells (Smallridge and Bull, 2000). The cytoplasm contains one, or rarely two, gamonts which usually do not displace the nucleus. The gamont is surrounded by a thick wall which may resist staining on a blood film. Ultrastructurally, the gamonts are seen within a parasitophorous vacuole in the erythrocyte cytoplasm. The thick wall or plasmalemma of the gamont contains suture points which typify the genus *Hemolivia* (Figure 5) (Beyer and Sidorenko, 1984; Smallridge and Paperna, 2000; Telford Jr., 2008).

Bobtail lizards transmit ticks horizontally when they are in a shared overnight shelter, and then become infected with *Hemolivia* following consumption of an infected tick (Bouma et al., 2007; Smallridge and Bull, 1999, 2000). Although minimal red cell pathology is associated with infection, higher parasite burdens have been associated with poorer body conditions and decreased activity levels in male bobtail, which was thought to be due to increased oxygen consumption (Main and Bull, 2000; Oppliger et al., 1996; Schall, 1986; Smallridge and Bull, 2000). In a study which collected *Amblyomma* ticks from adult bobtails
and examined preparations from tick gut contents, 34% of ticks contained *Hemolivia* oocysts. Results were compared to the presence or absence of parasitaemia on the blood smear from the bobtail host: only 13% of lizards had detectable parasitaemia. In addition, some infected lizards were identified which were not concurrently parasitised by ticks (Smallridge and Bull, 2001).

Figure 5: (Left) Bobtail erythrocyte infected with *Hemolivia mariae* (arrow). (1000 x magnification, Giemsa) Photographed with kind permission from Catherine Smallridge (Smallridge, 1998).
(Right) Transmission electron microscopic photomicrograph of longitudinally-sectioned *Hemolivia mariae* within an erythrocyte. Arrows indicate suture points in the capsule (n=nucleus). (9000x magnification) From (Smallridge and Paperna, 2000). Reproduced with kind permission from Catherine Smallridge.

The lifecycle of *Hemolivia mariae* is similar to that of the haemogregarine *Hemolivia stellata* which infects the toad *Bufo marinus* (Petit et al., 1990). When an *Amblyomma limbatum* tick attaches and feeds from a bobtail infected with *H. mariae*, it ingests the erythrocytes containing intracytoplasmic gamonts. These gamonts are released following the breakdown of erythrocytes within the tick gut, and the free gamonts enter the gut epithelial cells via a parasitophorous vacuole. Gamonts divide to produce a pair of microgametes. A microgamont fuses with a macrogamont to form the zygote (Figure 6). The zygote divides by sporogony and develops into a large (142-201µm), two to five-armed stellate oocyst (Figure 7). These stellate oocysts may become so large that they destroy the epithelial cell they are
within and are released into the gut lumen. Each oocyst produces hundreds of motile sporozoites. The sporozoites are released from the oocysts into the gut lumen. Free sporozoites then re-enter the gut epithelial cells and encyst as meronts containing 16-20 merozoites. Meronts may also be present within the gut lumen. A bobtail becomes infected with *H. mariae* following ingestion of an infected tick. The pre-patent period is 35-49 days, following which a parasitaemia is detectable. Merozoites are released from meronts in the tick gut within the gastrointestinal tract of the bobtail. Merozoites enter the cytoplasm of erythrocytes, appearing as a slim form with an indistinct nucleus. The merozoites become meronts, then enlarge and produce further merozoites. Merozoites are released from erythrocytes, infect other erythrocytes, encyst and mature to gamonts. After 70 days or more post-infection, the majority of the parasites are encysted gamonts with a faint blue polar nucleus. These gamonts may persist at low levels for up to 17 months. Gamont-containing erythrocytes are ingested by ticks that parasitise the infected bobtail (Figure 8). It is not known whether there is also a tissue phase of infection or if all merogony occurs solely in the erythrocytes of the bobtail, as no meronts have been demonstrated in histopathology tissue sections (Smallridge, 1998; Smallridge and Bull, 2001). In the related haemogregarine *Hemolivia stellata*, merogony occurs in the liver and spleen of the toad host (Lainson et al., 2007).

![Figure 6: A zygote in a gut smear from the tick *Amblyomma limbatum*. (Original magnification 200x, Giemsa) Photographed with kind permission from Catherine Smallridge (Smallridge, 1998).](image)
Figure 7: Star-shaped oocysts with 3, 4 and 5 arms in gut smears from the tick *Amblyomma limbatum*. (200x magnification, Giemsa) Photographed with kind permission from Catherine Smallridge (Smallridge, 1998).
Figure 8: *Hemolivia mariae* lifecycle. From (Smallridge, 1998). Reproduced with kind permission from Catherine Smallridge.
Leukocytes

Myelopoiesis

Myelopoiesis occurs primarily in the reptile bone marrow but has also been detected in the liver in late-stage embryos of the European pond turtle (Vasse and Beaupain, 1981). The maturation sequence of reptilian myeloid cells is similar to that in mammals and birds. Through maturation, the cells decreases in size, the nucleus condenses and the cytoplasm becomes progressively less basophilic (Campbell, 2004). Primary granules appear in the progranulocyte stage and the specific granules of the granulocytic myeloid cells appear in the myelocyte and metamyelocyte stages (Campbell, 2004; Campbell and Ellis, 2007; Mateo et al., 1984).

The normal total white cell count (WBC) of reptiles range from 5 to 15 x 10^9/L. Due to the diverse number of preanalytical factors that can influence reptilian leukocytes, it has been suggested that significance only be given to total or differential counts outside greater than twice the reference interval (Campbell, 2006). Leukocytosis is most often due to inflammation or infection. Reptiles can show stress leukograms, with the resultant total white cell count less than 20 x 10^9/L. Total white cell counts of greater than 30 x 10^9/L generally indicate bacterial sepsis, or rarely leukaemia (Tocidlowski et al., 2001). Lymphoid and myeloid leukaemia have been reported in lizards (Garner et al., 2004; Tocidlowski et al., 2001). Leukopenia may be found in overwhelming sepsis, and has been reported secondary to fenbendazole toxicity in the Hermann’s tortoise (Neiffer et al., 2005).

In most reptiles, the predominant circulating leukocyte is the lymphocyte, but some, namely those in the Scincidae family (skinks), are primarily heterophilic (Hawkey and Dennett, 1989; Strik et al., 2007). Leukocytes are broadly classified as granulocytes (heterophils, eosinophils and basophils) or mononuclear cells (monocytes, azurophils and lymphocytes).
Granulocytes
Heterophils

Morphology

Reptile heterophils are typically round to oval cells of 10 to 23µm in diameter. On Romanowsky stains, the cytoplasm appears colourless and contains pink granules that are rod-shaped, elliptical or ovoid, depending on the species (Campbell, 2006; Raskin, 2000). Heterophils may be seen in variable stages of degranulation on blood smears which is considered an artefact of sample handling, storage or fixation (Strik et al., 2007). The nucleus may be oval, bilobed or round, and usually eccentric (Campbell, 2006). Bilobed heterophils with sparse eosinophilic granules are the normal morphology seen in bobtails, and this morphology is considered typical of skinks (Hawkey and Dennett, 1989).

Ultrastructurally, reptile heterophils are round cells with a variably indented or lobulated nucleus (depending on the species) which contains a moderate amount of heterochromatin (Salakij et al., 2002; Work et al., 1998). The cytoplasm contains numerous round or rod-shaped granules. In several reptile species such as the loggerhead sea turtle, king cobra, the Hawaiian green turtle, the eastern diamondback rattlesnake and the black and white tegu lizard, two types of heterophil granules have been identified by transmission electron microscopy (Alleman et al., 1999; Carvalho et al., 2006; Casal et al., 2007; Salakij et al., 2002; Work et al., 1998). The larger granules are more electron-dense, and the smaller granules are variably but often less electron-dense. It has been postulated that the two granules may represent variable stages of maturity in circulation (Salakij et al., 2002). The reptile granules may be similar to the two types of granules identified in birds: the large electron-dense type corresponds to the eosinophilic granules seen on Wright’s-Giemsa stained blood films, and there are also smaller electron-dense, rod-shaped granules which are not seen by light microscopy (Montali, 1988).
**Function**

The reptile heterophil is functionally equivalent to the mammalian neutrophil, with response to inflammatory stimuli and infection as their primary role (Montali, 1988; Nabity and Ramaiah, 2010). Heterophils can phagocytose Gram positive and Gram negative bacteria as well as particles (Montali, 1988).

The substances found within heterophil granules as identified by cytochemical stains vary considerably between reptile species. Positive cytochemical reactions seen commonly in reptile heterophils include periodic acid-Schiff (PAS) (carbohydrates), Sudan black B (lipids), and the enzymes non-specific esterase, alkaline phosphatase, acid phosphatase, β-glucuronidase (Montali, 1988; Rovira, 2010). Most reptilian heterophils stain negative with the peroxidase cytochemical reaction due to a lack of the enzyme myeloperoxidase (Raskin, 2010). Myeloperoxidase is found in the primary granules of neutrophils in most mammals and functions in the oxygen-dependent microbial killing reaction known as the respiratory or oxidative burst (Campbell, 2006; Nabity and Ramaiah, 2010). Non-mammalian species (and rabbits) form caseous pus (heterophilic granuloma) rather than the liquid pus found in mammals due to this lack of heterophil myeloperoxidase (Myers et al., 2012; Raskin, 2010). Thus non-mammalian species rely on oxygen-independent mechanisms of microbial killing (Campbell, 2006). Lysozyme and acid phosphatase, important for antimicrobial activity, are also found in reptile heterophils (Claver and Quaglia, 2009; Harmon, 1998; Zimmerman et al., 2010a). Other molecules found within heterophils of birds which may also be present in those of reptiles is the acid hydrolase α-glucosidase, as well as antimicrobial β-defensins and the protease cathepsin (Harmon, 1998; Zimmerman et al., 2010a).

**Laboratory evaluation**

The number of circulating heterophils is highly dependent on the season and the species. Heterophil numbers are highest in summer and lowest during brumation (Campbell, 2006). Most skinks have high numbers of circulating heterophils, for example, in the prehensile-
tailed skink, heterophils comprised 18 to 68% (mean 36%) of the circulating leukocytes in summer (Hawkey and Dennett, 1989; Wright and Skeba, 1992).

**Abnormalities**

Heterophilia may be present in stress, inflammation, bacterial or fungal infections, neoplasia and myeloid leukaemia (Campbell, 2006). If inflammation or infection exhausts the bone marrow reserve of mature heterophils, a left shift will be seen, as in mammals. Immature heterophils are characterised by the presence of a larger nucleus and purple (basophilic) primary granules (Strik et al., 2007). Inflammation or infection may also result in toxic changes including cytoplasmic basophilia, abnormal granulation, or cytoplasmic vacuolation. Where it is not a normal feature of the heterophils of that species, nuclear lobulation is also considered a toxic change (Campbell, 2006).

Heteropenia can be seen secondary to overwhelming sepsis or viral diseases such as inclusion body disease in boas (Campbell, 2006).

**Eosinophils**

**Morphology**

Reptilian eosinophils range from 9 to 20µm in diameter and exhibit variable morphology between species (Raskin, 2000). The nucleus is generally round to oval and central, though it may be lobed in some species. The cytoplasm is typically clear, but may stain pale blue on Romanowsky stains in species such as iguanas. The cytoplasm contains granules which are often more rounded than those of heterophils, and they may stain bright pink, dull pink or pale blue with Romanowsky stains (Campbell, 2006).

Under transmission electron microscopy, reptile eosinophils are round cells with a round to oval nucleus and variable amounts of heterochromatin. They contain numerous, large,
electron-dense granules in the cytoplasm, and in some species such as the Hawaiian green turtle and the black and white tegu lizard, the granules contain elongate crystalline structures, similar to those seen in many mammalian species (Carvalho et al., 2006; Work et al., 1998). In mammals, this crystalline material within eosinophil granules is major basic protein (Cheville, 2009). Vacuoles may also be seen within the cytoplasm (Casal et al., 2007; Work et al., 1998; Zhang et al., 2011).

Function

The function of reptile eosinophils appears to be phagocytosis of immune complexes formed during parasitic infections (Sypek and Borysenko, 1988). Cationic proteins have been identified in the eosinophils of some lizards, which are important for parasite killing (Carvalho et al., 2006). Phagocytic and microbicidal activity have been reported in eosinophils of the American alligator (Mateo et al., 1984).

As with heterophils, the cytochemical staining properties of reptile eosinophils are highly variable between species (Campbell, 2006). Eosinophil granules may stain with PAS, alkaline phosphatase or peroxidase (Raskin, 2010). The peroxidase present in eosinophils is biochemically and structurally different to that found in heterophils, but is also capable of causing oxidative damage (Jain, 1986; Raskin, 2010).

Laboratory evaluation

Eosinophil numbers are generally lowest in summer and highest during brumation. Most lizards have low numbers of circulating eosinophils, and some snake species lack eosinophils (Campbell, 2006). Normal turtles may have up to 20% circulating eosinophils (Campbell, 2004).
Abnormalities

Eosinophilia may be caused by inflammation or parasitism, e.g., by protozoa and helminths, (not haemoparasites) (Campbell, 2004, 2006). The significance of eosinopenia in reptiles is unknown (Strik et al., 2007).

Basophils

Morphology

Basophils are small, 7 to 20µm diameter cells with a round, typically central nucleus. On Romanowsky-stained smears, the cytoplasm contains numerous large purple granules which often obscure the nucleus (Sypek and Borysenko, 1988).

The ultrastructure of reptile basophils is dominated by numerous, homogenous, electron-dense cytoplasmic granules (Carvalho et al., 2006; Salakij et al., 2002). The nucleus may be central or eccentric, with peripheral heterochromatin (Carvalho et al., 2006).

Function

The role of basophils in reptiles appears to be surface immunoglobulin expression and release of histamine as part of the immune response (Mead et al., 1983).

Toluidine blue is a basic dye which reacts with acid mucopolysaccharides such as heparin (Raskin, 2010). The basophil granules of alligators, desert tortoises and some lizards stain with acidic toluidine blue, suggesting the granule contents include heparin (Alleman et al., 1992; Jain, 1993; Mateo et al., 1984). Alcian blue, another stain which reacts with mucopolysaccharides, may also stain reptile basophils. Other substances which may be found in reptile basophils, as identified by cytochemical stains, include acid phosphatase and glycogen (Raskin, 2010).
Laboratory evaluation

Basophil percentages are highly variable between reptile orders, with some chelonians possessing up to 60% circulating basophils (Claver and Quaglia, 2009). Basophil numbers do not seasonally fluctuate (Campbell, 2006).

Abnormalities

Basophilia is usually due to infection with haemoparasites such as haemogregarines and trypanosomes, viruses e.g., iridoviruses or intestinal parasites (Campbell, 2006; Sypek and Borysenko, 1988).

Mononuclear cells

Monocytes and azurophils

Morphology

Monocytes are large leukocytes, 8 to 20µm in diameter, with a similar morphological appearance to those of mammals. The nucleus is round to oval, or may be lobed, and the cytoplasm is usually pale blue on Romanowsky-stained smears, and may contain vacuoles (Campbell, 2006). Some reptiles have azurophils. Azurophils are large mononuclear cells, of a similar appearance to monocytes, with a dusting of fine pink/purple granules in the cytoplasm. They are considered by most authors to be a monocyte subtype (Campbell, 2006; Campbell and Ellis, 2007).

When examined using a transmission electron microscope, monocytes are round cells with a round to indented nucleus. The nucleus contains a variable amount of heterochromatin (Casal et al., 2007; Martinez-Silvestre et al., 2005). Within the cytoplasm there are variable numbers of small, electron-dense granules (Carvalho et al., 2006; Casal et al., 2007; Salakij et al., 2002; Work et al., 1998). In the giant lizard, monocytes can be ultrastructurally differentiated from azurophils by the nuclear to cytoplasmic ratio: monocytes have a much
higher N:C ratio than azurophils. Monocytes also possess fewer granules (Martinez-Silvestre et al., 2005; Salakij et al., 2002).

**Function**

Monocytes and macrophages are important phagocytic cells that kill microbes by releasing reactive oxygen and nitrogen species. Opsonisation, or the coating of a microbe with immunoglobulins or complement, enhances macrophage killing in reptiles as in higher vertebrates. The reptile macrophage oxidative burst is highly temperature-dependent, being more effective at 35°C than 30°C and below (Pasmans et al., 2001). Ectotherms are not capable of producing pyrexia but will actively seek heat to increase their body temperature during infection in order to enhance macrophage function (Pasmans et al., 2001; Zimmerman et al., 2010a).

Substances found within the cytoplasm and granules of monocytes and azurophils, identified by cytochemical stains, are highly variable between species. In snakes, azurophils appear to have a different function to monocytes – the cytochemical staining characteristics are different to monocytes and are more similar to the mammalian neutrophil (i.e., peroxidase and Sudan black positive). Similarly, snake azurophil numbers tend to increase during acute inflammation. This is not usually the case for lizards, with similar cytochemical staining and therefore assumed similar function of azurophils to mammalian monocytes (i.e., peroxidase and Sudan black negative, acid phosphatase positive). Consistent with this, an increase in azurophil numbers is typically seen in lizards during chronic inflammation (Stacy et al., 2011).

**Laboratory evaluation**

Monocyte numbers do not appear to fluctuate seasonally (Raskin, 2000). Reptiles usually have less than 10% monocytes and azurophils (Sypek and Borysenko, 1988). Azurophils are commonly found in reptiles of the order Squamata, particularly snakes (Montali, 1988).
**Abnormalities**

Monocytosis and/or azurophilia may be seen with inflammation, particularly granulomatous disease (Campbell, 2006; Strik et al., 2007). Reptiles form histiocytic granulomas in response to intracellular pathogens (Montali, 1988).

In reptiles, peripheral erythrophagia by monocytes may be seen in anaemia, and cytophagia of other leukocytes may be seen during inflammation (George et al., 2008). Azurophils exhibiting erythrophagia have been reported on blood smears from a bearded dragon and a python, and the erythrophagia was presumed to be secondary to myeloproliferative neoplasia and a viral infection respectively (Jaensch and Raidal, 2006).

**Lymphocytes**

**Lymphopoiesis**

Lymphocytes are produced by the bone marrow and spleen. In the spleen, lymphopoiesis begins several weeks before birth and the final splenic structure is established just prior to birth/hatching (El Ridi et al., 1988). The spleen contains white pulp centred around arterioles as in mammals, red pulp, as well as a marginal zone for cell trafficking (El Ridi et al., 1988; Zimmerman et al., 2010a). True germinal centres are not found in the spleen of reptiles (Bao et al., 2009).

The structure of the thymus and the thymic lymphocyte population are established just before birth/hatching and then they regress with age. Lymphocytes enter the thymus and differentiate into T cells. The reptile thymus is a similar structure to the mammalian thymus, with the cortex containing lymphocytes and the medulla containing lymphocytes and epithelial cells (Hassall’s corpuscles) (El Ridi et al., 1988).

The site of B cell development in reptiles is currently unknown, as an equivalent to the avian bursa of Fabricius has not been identified (Campbell and Ellis, 2007). Zones within haematopoietic tissues are postulated as possible sites (Boehm et al., 2012).
Morphology

Reptilian lymphocytes are morphologically very similar to those found in mammals, with a large round nucleus and pale blue cytoplasm (Campbell, 2006). They may be small, medium or large, ranging from 5 to 15 µm in diameter, with the small subtype the most numerous (Strik et al., 2007). Larger lymphocytes tend to have a lower nuclear to cytoplasmic ratio than small lymphocytes (Campbell, 2006). Small purple cytoplasmic granules may occasionally be seen. Particularly in lizards, it can be difficult to differentiate lymphocytes from thrombocytes on a blood smear and also in a haemacytometer counting chamber. Differentiation may be assisted with cytochemistry: lymphocytes typically stain negative with cytochemical stains, and thrombocytes of many reptiles stain positive with periodic acid-Schiff (Strik et al., 2007).

Plasma cells may also be seen occasionally in the peripheral blood of reptiles and appear similar to those found in mammalian tissues (Campbell, 2006). Circulating plasma cells may rarely possess immunoglobulin-containing Russell bodies (Mott cells) (Reagan et al., 2008).

The ultrastructure of reptile lymphocytes is typified by a round to indented nucleus containing a moderate to large amount of heterochromatin, and a scant amount of cytoplasm with few organelles (Martinez-Silvestre et al., 2005; Salakij et al., 2002; Work et al., 1998). Small electron-dense granules may be present in the cytoplasm (Casal et al., 2007; Zhang et al., 2011).

Function

Lymphocyte function in reptiles is similar to mammals (Campbell, 2006). Neonatal reptiles acquire maternal immunity via the yolk. Maternal antibodies are detectable soon after birth and may persist for up to 12 months (Schumacher et al., 1999; Ward, 2004). The age of the development of a competent immune system varies between reptile species, with some fully functional from birth and others requiring several months to develop (El Deeb and Saad, 1990). In addition to the lymphoid tissue in the bone marrow, spleen and thymus, mucosal-associated lymphoid tissue is found in the respiratory and gastrointestinal tracts, (Claver and Quaglia, 2009; Zimmerman et al., 2010a). Most reptiles do not have lymph
nodes, but the bobtail lizard and snapping turtle have been reported to possess rudimentary nodes (Borysenko and Cooper, 1972; Wetherall and Turner, 1972).

Both B cells and T cells have been identified in reptiles. T cells function in cell-mediated immunity. As in mammals, reptile T cells can differentiate into cytotoxic T cells for direct killing, T helper cells or T regulatory cells. The T cell response is strongly seasonal, being lowest in winter (brumation) and maximal in spring/summer (El Ridi et al., 1988).

Reptile B cells produce antibodies or immunoglobulins in response to cytokines released by T cells. Reptile immunoglobulins (Ig) have the same basic structure as for mammals, with rearrangement of V, D, and J gene segments responsible for antibody diversity (El Ridi et al., 1988). Reptiles produce three types of immunoglobulins. IgM is the first-line response to infection which activates complement. Then IgY is produced several weeks later, which has a longer half-life and is more effective (Jacobson and Origgi, 2002; Turchin and Hsu, 1996; Wetherall, 1969; Zimmerman et al., 2010a). It has been postulated that mammalian IgG and IgE evolved from IgY (Warr et al., 1995). A third antibody, IgD has been identified in reptiles, however its function has not yet been elucidated (Wei et al., 2009). The presence of IgA has been reported in the small intestine of the leopard gecko but was not found in the genome of the anole lizard; it is thought that an increase in IgM secretion compensates for a lack of IgA in some reptiles as in fish and humans (Deza et al., 2007; Wei et al., 2009).

Reptile B cells start to produce antibodies one week after an antigen challenge, with peak levels 6-8 weeks post-infection at preferred body temperature (Wetherall and Turner, 1972; Zimmerman et al., 2010b). After a second challenge, the latent period is reduced but there is typically no increase in antibody binding affinity or antibody titres as in the typical mammalian response. This has been attributed to the lack of germinal centres in reptile lymphoid tissue (Hsu, 1998). Thus the response to vaccination would be much more variable in reptiles than in mammals, however vaccines have been reported as efficacious in controlling outbreaks of mycoplasmosis in farmed crocodiles (Mohan et al., 1997).
The presence of natural antibodies in reptiles has been postulated as a mechanism to compensate for the slow humoral response. A small subset of B cells, called B-1 cells, produce poly-reactive natural antibodies without requiring antigenic stimuli (Zimmerman et al., 2010b).

IgM-positive phagocytic B cells have been identified in fish, amphibians and recently reptiles, supporting a hypothesis that B cells evolved from phagocytic cells (Zimmerman et al., 2010b). Their role in phagocytosis *in vivo* is unknown.

**Laboratory evaluation**

Lymphocytes are the predominant leukocyte in many reptiles; up to 72% of the differential count in the Hawaiian green turtle (Work et al., 1998). Lymphocyte numbers are lowest in winter due to thymic and splenic white pulp involution, and increase again in the spring (El Ridi et al., 1988). Female reptiles tend to have higher lymphocyte counts than males (Sypek and Borysenko, 1988). Plasma cells are rare in circulation, usually less than 0.5% of the leukocyte differential (Strik et al., 2007).

**Abnormalities**

Lymphocytosis in reptiles may be due to inflammation, wound healing, parasitic infections, or viral diseases (Campbell, 2006). Leukaemia has been occasionally reported in several species of reptiles including lizards, with lymphoid leukaemia most frequently reported, either with or without concurrent lymphoma (Garner et al., 2004; Hernandez-Divers and Garner, 2003; Suedmeyer and Turk, 1996). Mild to marked lymphocytosis can also be seen in the inclusion body disease of boas and pythons (Campbell, 2006).

Lymphopenia may be due to endogenous corticosteroids due to malnutrition or other stress, or exogenous corticosteroids (Campbell, 2006). Reactive lymphocytes, possessing a more basophilic cytoplasm, and/or a concurrent increase in circulating plasma cells may be seen associated with antigenic stimulation (Campbell, 2006; Strik et al., 2007).
**Thrombocytes**

**Thrombopoiesis**

Thrombocytes are the reptilian equivalent of the mammalian platelet (Scott and Owens, 2008). Unlike mammals where platelets are produced from fragments of the polyploid megakaryocyte, thrombocytes are derived from a mononuclear progenitor cell, the thromboblast, which is similar in appearance to the rubriblast (Campbell, 1988; Campbell and Ellis, 2007; Tavassoli, 1980). The thromboblast then gives rise to early, mid and late-immature thrombocytes, also known as prothrombocytes, then mature thrombocytes (Campbell, 1988; Tadjalli et al., 1997). Each of the four stages becomes successively smaller, with a lower nuclear to cytoplasmic ratio. Eosinophilic granules may be seen in the cytoplasm from the mid-immature stage (Campbell, 1988).

As many as 16 thrombocytes may be produced from a single thromboblast by mitosis. In contrast, one mammalian megakaryocyte can produce up to 3000 platelets by mitosis and endomitotic polyploidisation. With the larger body sizes, higher metabolic rates, higher blood pressures and more complex vascular systems found in mammals, there is a greater demand for effective haemostasis. The evolutionary development of the mammalian megakaryocyte allowed a significant increase in the efficiency of the use of genetic material and efficacy of haemostasis (Schneider and Gatterman, 1994). From this, one researcher has posed the question whether dinosaurs had megakaryocytes or thrombocytes in their bone marrow (Brass, 2005).

**Morphology**

Mature thrombocytes are small, round to spindle-shaped nucleated cells, approximately 8 to 16µm long and 5 to 9 µm wide (Raskin, 2000; Sypek and Borysenko, 1988). They have a moderate to high nuclear to cytoplasmic ratio, with a central nucleus and dense chromatin pattern. The cytoplasm stains colourless to pale blue with Romanowsky stains, and may contain small vacuoles or granules (Campbell, 2006). Thrombocytes may be confused with lymphocytes, particularly in species where thrombocytes are round. A periodic acid-Schiff
(PAS) reaction may assist differentiation as thrombocytes tend to stain PAS-positive and lymphocytes PAS-negative in most species (Harr et al., 2001).

Thrombocytes readily become activated and form clumps in vitro, particularly when heparin is used as the anticoagulant (Campbell, 2006). The thrombocyte shape is maintained by a marginal band of microtubules, similar to that found in nucleated erythrocytes and mammalian platelets (Edmonds, 1968). When thrombocytes are activated, their cytoplasm is more likely to contain vacuoles, and the cell membrane often appears irregular (Campbell, 2004).

Ultrastructurally, the thrombocytes of many reptiles appear similar. They are oval to fusiform cells with an irregular, oval to lobulated nucleus with a moderate to large amount of heterochromatin. As in the platelets of many mammals, thrombocytes possess a canalicular network (Cheville, 2009; Daimon et al., 1987; Edmonds, 1968). In contrast to platelets, thrombocytes contain a full complement of cellular organelles including a nucleus, Golgi apparatus and rough endoplasmic reticulum. Few small, electron-dense granules may be seen. In some thrombocytes, the cell membrane is folded into finger-like projections (Carvalho et al., 2006; Casal et al., 2007; Work et al., 1998; Zhang et al., 2011).

**Function**

Thrombocytes have a short lifespan in vitro with degeneration evident after 3 hours. Thrombocytes function in a similar way to mammalian platelets, with haemostasis their primary role (Wigley et al., 1999).

In turtles, thrombocyte adhesion to damaged subendothelial collagen is facilitated by binding of proteins similar to the mammalian von Willebrand’s factor (vWF) and platelet glycoprotein Ib (Soslau et al., 2005). Reptile thrombocyte aggregation can be induced by
collagen (contact) and thrombin, but not ADP as thrombocytes lack ADP receptors (Schneider and Gatterman, 1994; Soslau et al., 2005). Thrombocyte aggregation in birds is less efficient than that of mammals due to lower surface expression of glycoprotein IIb-IIIa (Schmaier et al., 2011). This may also be true for reptiles but to the author’s knowledge, no studies have been published on this.

Reptile thrombocytes may release granule contents through the canalicular system. Structures similar to mammalian alpha granules have been reported in the tortoise thrombocyte: in mammals, the contents of alpha granules include fibrinogen, von Willebrand’s factor, thrombospondin and platelet-derived growth factor (Boudreaux, 2010; Daimon et al., 1987). Snake thrombocytes contain structures which were identified as dense granules but at least one species of turtle does not; mammalian dense granules contain serotonin, calcium and adenine nucleotides (Boudreaux, 2010; Daimon et al., 1987). Alligator, snake and some turtle thrombocytes contain serotonin (Maurer-Spurej, 2005). Reptile thrombocytes do not store or secrete adenine nucleotides (Schneider and Gatterman, 1994).

The coagulation times of reptiles in vitro are longer than that of mammals, with one study on iguanas reporting variable coagulation times depending on the (mammalian) reagents and methodology used, but the overall median prothrombin time (PT) was 11 minutes and median activated partial thromboplastin time (APTT) was 3 minutes (Kubalek et al., 2002). In a study of coagulation in various species of sea turtles, the PT was approximately 44 seconds and the APTT was greater than 10 minutes (Soslau et al., 2004). Soslau et al. (2004) also looked at individual coagulation factors in turtles and concluded that they appear to lack a complete intrinsic (contact) coagulation pathway, specifically factors XI and XII. This is also true for fish and birds, but not for amphibians (Ponczek et al., 2008). Although the suitability of mammalian coagulation tests for reptiles is questionable, and coagulation is highly temperature-dependent in reptiles, the slow clotting seems to be at least partially attributable to an as yet uncharacterised natural anticoagulant in the plasma of reptiles (Hackett and Hann, 1967; Kubalek et al., 2002). Whole, untreated blood from *Tiliqua rugosa*...
takes more than 60 minutes to clot in vitro. The hypocoagulability of reptile blood is thought to be an adaptation to minimise thrombosis with the low blood pressures, slow blood flow and metabolism of reptiles (Hackett and Hann, 1967).

In addition to haemostasis, thrombocytes appear to have multiple functions in innate immunity (Wigley et al., 1999). Reptile thrombocytes are capable of phagocytosing Gram positive and Gram negative bacteria (Strik et al., 2007; Taffarel and Oliveira, 1993; Wigley et al., 1999). They possess lysosomes which contain acid phosphatases, providing supporting evidence for their phagocytic role (Daimon et al., 1987). Thrombocytes may also phagocytose red blood cells, particles and dyes, thus phagocytosis by these cells is considered non-specific (McDaniel et al., 1987; Wigley et al., 1999). In chickens, thrombocytes can be involved in the inflammatory response, with production of interleukin-6 and prostaglandin E2 to lipopolysaccharide via Toll-like receptor-4 activation (Scott and Owens, 2008). The involvement of reptile thrombocytes in inflammation is not well understood.

**Laboratory evaluation**

Thrombocyte clumping in reptile blood samples often precludes the ability to produce accurate thrombocyte counts (Campbell, 2006). Reptiles usually have between 25 and 350 thrombocytes per 100 leukocytes (Harr et al., 2001). A few species such as the Brazilian boa constrictor have been reported to have an increased thrombocyte count in summer (Machado et al., 2006).

**Abnormalities**

Thrombocytopenia may be caused by decreased production or increased utilisation of thrombocytes (Harr et al., 2001; Strik et al., 2007). Prior to further investigation of thrombocytopenia, a falsely low thrombocyte count due to clumping must be ruled out. Decreased thrombocyte production may be due to infection, toxins or bone marrow
neoplasia. Increased utilisation of thrombocytes may occur with infection, inflammation, or immune-mediated disease (Strik et al., 2007). Lobulated nuclei may be seen in thrombocytes during severe inflammatory disease, and also in reptiles with chronic anorexia (Hawkey and Dennett, 1989).

**Total plasma protein**

Strictly speaking, total plasma protein is not considered a component of haematology, but it is commonly included in the complete blood count as it is routinely measured on centrifuged microhaematocrit tubes following measurement of the PCV. Very little is different in the consideration of total plasma protein in reptiles as compared to mammals.

Total plasma protein can be calculated by measuring the refractive index of centrifuged plasma on a handheld refractometer. The refractive index reflects the concentration of all solids in plasma, the vast majority of which are proteins. In healthy animals, proteins are chiefly albumin or globulins, with very small contributions made by other proteins such as fibrinogen and other coagulation factors. As in mammals, the presence of large numbers of other solids in the plasma as seen with lipaemia and haemolysis will falsely elevate the total plasma protein in reptiles.

Total plasma protein concentrations of healthy reptiles typically range from 30 to 70g/L (Campbell, 2006). In female reptiles during active folliculogenesis, the total plasma protein may be physiologically increased due to an increase in globulin synthesis for yolk production.

Hyperproteinaemia is commonly associated with dehydration.

Hypoproteinaemia may be seen with decreased protein intake such as chronic malnutrition, malabsorption or maldigestion. Hypoproteinaemia may also be due to increased protein loss from protein-losing enteropathy e.g., due to gastrointestinal parasitism, severe blood loss, or renal disease. Finally, hepatic insufficiency can cause decreased production of
albumin, resulting in hypoproteinaemia (Campbell, 2006; Diethelm and Stein, 2006; Proverbio et al., 2012).

There are many features of reptile haematology which differ from those of mammals, and these need to be considered when evaluating results.
Reference intervals

In clinical pathology, reference intervals are formed in an attempt to define the physiological variation of a parameter for a specific set of conditions (Friedrichs et al., 2011). A result which falls outside this interval is therefore classified as abnormal; and whether this is considered clinically significant or not is determined by the clinical pathologist or the clinician. The Reference Value Advisor freeware macro for Microsoft® Excel® has been developed by Geffré et al. (2011) as a tool to construct reference intervals specifically for veterinary clinical pathology. This software has been designed to produce reference intervals in accordance with the American Society of Veterinary Clinical Pathologists (ASVCP) Quality Assurance and Laboratory Standards Committee (QALS) Guidelines for the Determination of Reference Intervals in Veterinary Species (Friedrichs et al., 2011). The QALS guidelines have themselves largely drawn on the recommendations from the Clinical and Laboratory Standards Institute and International Federation of Clinical Chemistry (CLSI-IFCC) guidelines which were established for human medicine (Clinical and Laboratory Standards Institute, 2008). Reference intervals are required for meaningful interpretation of haematology data.
Published research on the haematology of bobtail lizards

Only one study has been published on the haematology of *Tiliqua rugosa*. Canfield and Shea (1988) collected blood into K$_2$EDTA from the ventricle of six bobtails, as well as three blue-tongue lizards (*Tiliqua scincoides*), and one bobtail/bluetongue hybrid, at various times of the year (seasons not further specified). They performed red blood cell and white blood cell counts using the Unopette® dilution system and a haemacytometer, and examined Giemsa-stained blood smears by light microscopy. Haemoglobin was determined using a Royco 720-A Hemoglobinometer following lysis (method not specified) and centrifugation. Blood cells from a microhaematocrit buffy coat were fixed and examined by transmission electron microscopy.

**Haematology data**

The mean PCV of bobtails was 0.26L/L, mean red blood cell count 0.95 x10$^{12}$/L, haemoglobin 68g/L, white blood cell count 4.07 x10$^9$/L, heterophils 2.49 x10$^9$/L (61%), lymphocytes 0.24 x10$^9$/L (6%), monocytes (including azurophils) 1.20 x10$^9$/L (29%), eosinophils 0.07 x10$^9$/L (2%), basophils 0.08 x10$^9$/L (2%), and thrombocytes 20.67 x10$^9$/L. The plasma was light yellow with a mean total plasma protein (by refractometry) of 47g/L (Canfield and Shea, 1988).

From this data, it was concluded that the predominant leukocyte in the bobtail is the heterophil, and they have low numbers of circulating lymphocytes, eosinophils and basophils. The low lymphocyte count differs from that encountered in the prehensile-tailed skink (22%) and *Egernia* skink (10-40%), which are the most closely related lizards with published leukocyte differential counts (Cartledge et al., 2005; Wright and Skeba, 1992). The basophil percentage was also higher in the prehensile-tailed skink (15%) (Wright and Skeba, 1992). Compared to data from other reptiles, the PCV, red blood cell count, and haemoglobin concentration are within the expected ranges (0.2-0.4L/L, 1.1.5 x 10$^{12}$/L, and
60-100g/L respectively). The total white blood cell count of bobtails is slightly lower than the expected range of 5 to $15 \times 10^9$/L. The thrombocyte count is most often given as a count on a blood smear compared to the number of white blood cells, with normal given as 25 to 250/100 WBC (Campbell, 2006; Harr et al., 2001). In this case, converting the above data, the thrombocyte count would be 500 thrombocytes per 100 white blood cells, above the suggested range. Thrombocyte counts are highly variable due to their proclivity for clumping in vitro.

The data from Canfield and Shea (1988) are generally in good agreement with reference ranges from other reptiles and lizards. However, as the season(s) the blood collections were performed in was not provided, comparison of data in the present study to these values may not be appropriate.

**Morphology**

On light microscopy, mean erythrocyte measurements were 17.5µm x 9.1µm, with mature and immature erythrocytes described as typical for lizards. Immature red blood cells were seen on all Giemsa-stained smears (the degree of polychromasia was not described). Ultrastructurally, the erythrocytes had a grey homogenous cytoplasm with marginal bands of microtubules.

The heterophils were described as round cells, 15.3µm in diameter, with an eccentric oval to variably lobulated nucleus with clear to slightly grey cytoplasm, variable numbers of vacuoles and round to oval, variably-sized, orange-brown granules. Some cells were devoid of granules and were uniformly vacuolated. The ultrastructural features included a highly irregular nucleus, the lobes of which often appeared disparate, with dense heterochromatin. The cytoplasm was densely packed with many variably-sized, round to oval to elongate dense granules, as well as organelles. Some granules were vacuolated.
Eosinophils were described as round cells, 16.4µm in diameter, with densely-packed purple to red, round to oval granules which often covered the nucleus. The nucleus was oval to round and eccentric. Transmission electron microscopic examination revealed an oval, eccentric nucleus with highly variable-appearing granules, some of which contained a crystalline inclusion, and others containing vacuoles.

Basophils were 12.7µm in diameter with a round central nucleus which was often obscured by many round, purple to blue cytoplasmic granules. Ultrastructurally, the nucleus was round and the cytoplasm contained large, round, dense, homogenous granules, as well as low numbers of smaller, dense granules.

Monocytes were described as round cells with an oval to round nucleus, and often many fine azurophilic granules in the cytoplasm and a mean diameter of 13.1µm. On the transmission electron microscope, the bobtail monocytes had mildly irregular nuclei with small amounts of heterochromatin. Many organelles were seen within the cytoplasm, and vacuoles and oval to elongate, variably-sized, dense granules were also present.

Lymphocytes possessed a round nucleus with fine chromatin, and variable quantities of pale blue cytoplasm with occasional vacuoles or granules. The ultrastructure was typified by a round to slightly indented nucleus with prominent clumps of heterochromatin, and occasional dense granules in the cytoplasm.

Thrombocytes were oval to round cells, often with cytoplasmic vacuolation, and seen in clusters. Length to width measurements were 7.5 x 9.3µm. Thrombocytes could not always be readily distinguished from lymphocytes by light microscopy. On transmission electron microscopy, thrombocytes possessed an irregular nucleus with dense heterochromatin. The cytoplasm contained small dense granules and vacuoles (Canfield and Shea, 1988).
The morphology of all peripheral blood cells described by Canfield and Shea (1988) are similar to descriptions provided for other lizards, and skinks in particular, however, only six lizards were used in Canfield and Shea’s study (Hawkey and Dennett, 1989). This sample size is too small to extrapolate haematology reference intervals. Also, the seasons in which blood was collected were not specified. Season, as well as many other preanalytical factors, may greatly influence reptile haematological data. Thus a larger sample size with controlled preanalytical factors, including season, is required in this study to produce valid haematology reference intervals.
Respiratory disease in reptiles

In this study, reference intervals will be developed for the haematology of healthy adult bobtails and compared to the haematology of bobtails with upper respiratory tract disease. Little is known about the aetiopathogenesis of this upper respiratory disease. It is thus useful to consider the normal structure and function of the reptilian respiratory tract, and the common causes of respiratory disease in other reptiles.

Normal respiratory tract structure and function

The upper respiratory tract functions to cleanse, humidify and then transport air to the lungs to facilitate gas exchange. Reptiles have paired nasal sinuses lined by mucous epithelium ventrally and multi-layered olfactory epithelium dorsally which meet caudally in the choana.

The entrance to the larynx and trachea, the glottis, is further rostral than the oesophagus, and is located on the ventral floor of the buccal cavity which can be visualised readily upon opening the mouth. The trachea is formed by C-shaped cartilage rings, and it bifurcates in the cranial coelom at the heart base to the mainstem bronchi, then further splitting into secondary bronchi. The trachea and bronchi are lined by ciliated epithelium, secretory and basal epithelial cells (Schumacher, 1997).

Grossly, the lungs are long thin membranous sacs. Reptile orders differ in their lung morphology, with most snakes having only one functional lung (Schumacher, 1997). Most lizards, including bobtail lizards, have paired lungs of equal sizes. The surface area for gas exchange of the reptile lung is 10 to 20% that of mammals, which is considered in keeping with the 10 to 33% lower basal metabolic rate of reptiles (Jacobson, 2007).
The lungs function in gas exchange. Most reptiles including the bobtail lizard do not have a diaphragm so they rely on abdominal muscles to inflate the lungs, and they cannot cough to expel material from the airways. Bronchi terminate into a central lumen, from which there are many invaginations known as faveolae and ediculae, separated by connective tissue septae with a small luminal bundle of smooth muscle, and occasional lymphoid aggregates. The faveolae and ediculae are lined by a single layer of type I squamous epithelial cells and type II cuboidal epithelial cells which contain punctate granules. Some reptiles also have hedge cells at the junction of the ciliated respiratory cells and the type I and II cells. Hedge cells possess microvilli and are thought to be involved in resorption of fluid. Beneath the type I cells are the thin-walled capillaries of the interstitium. Scattered neuroendocrine cells are also found between epithelial cells either singly or in clusters (Schumacher, 1997).

**Causes of upper respiratory tract disease in reptiles**

The typical signs of upper respiratory tract disease represent aberration of the normal cleansing and humidifying functions of the region. The signs may include sneezing, watery ocular and nasal discharge, or mucoid, tenacious and adherent ocular, nasal and oropharyngeal material.

Various viruses can cause respiratory disease in reptiles including herpesviruses, paramyxovirus, ranavirus, reoviruses, and (retrovirus-like) inclusion body disease of boids (Ariel, 2011; Jacobson et al., 1997; Laboklin, 2008; Schumacher, 2006; Westhouse et al., 1996). Diagnosis is typically by PCR of swabs or tissues; serological testing is available for a select few viruses (Ariel, 2011; Laboklin, 2008; Schumacher, 2006).

*Mycoplasma agassizii* causes chronic upper respiratory tract disease in tortoises (Jacobson et al., 1991). Infected tortoises present with serous to purulent ocular and nasal discharge. There is sometimes bubbling of the discharge, accompanying conjunctival and nasal oedema, and inflammation (Brown et al., 1999; Brown et al., 1994). Diagnosis of
mycoplasmosis is by ELISA or PCR from nasal flush fluid, oral or nasal swabs, or from fresh tissues (Laboklin, 2008; Schumacher, 2006).

With the exception of Mycoplasma agassizii infection in tortoises, aerobic and anaerobic bacterial infections of the respiratory tract are generally opportunistic or secondary. Similarly, fungal infections can also occur and are usually opportunistic or secondary. Infections are usually associated with immunosuppression due to improper husbandry or concurrent disease (Schumacher, 1997).

Various parasites as well as non-infectious aetiologies such as foreign bodies, trauma, neoplasia and inhalation of toxic or irritant agents may also cause respiratory tract disease in reptiles (Schumacher, 1997, 2011).

**Upper respiratory tract disease in bobtail lizards**

Since 1996, hundreds of wild bobtail lizards have been taken to wildlife rehabilitation centres in Perth with “bobtail flu” (Haight, 2013). These lizards typically present in summer, are dull and quiet, with a serous to mucoid ocular and nasal discharge which may bubble and can glue the eyelids closed and occlude the nares. Frequent sneezing is also common, and plugs of thick, clear, tenacious mucus may be seen in the caudal choana. Oral mucous membranes may be of variable pallor but are often pale pink. Dehydration is usually present and body condition is often decreased, seen as decreased tail fat stores and a prominent pelvis. The illness affects adult, juvenile and neonate bobtails. The disease is anecdotally infectious which has led to the establishment of separate isolation wards for affected bobtails at rehabilitation centres. Neonates born in hospital to affected mothers are said to develop clinical signs if they are not separated from the mother’s vivarium within a day or so after birth (Haight, 2013). This suggests that if an infectious organism is responsible, transplacental infection is unlikely. Upper respiratory tract infections are typically spread by aerosolisation or direct mucous membrane contact with infected discharge e.g., from sharing food or water bowls. Prevention of the development of clinical signs in these
removed neonates may support this mode of infection. Similar clinical signs have also been seen in two wild bluetongue lizards and one king skink (Kristy Gaikhorst, pers. comm.).

Preliminary and unpublished research has been conducted by Dr. Mark Bennett, Dr. Tim Hyndman and Brett de Poister at Murdoch University to investigate potential infectious causes of this disease. PCR was performed to detect herpesviruses and adenoviruses from ocular, choanal and cloacal swabs (n=21). Archived post mortem reports and histopathology slides were reviewed from four bluetongue lizards and 12 bobtails, including four bobtails with clinical signs of “flu” from Murdoch University and one from the Department of Agriculture and Food Western Australia (DAFWA). No causative agent could be identified. The bobtail from DAFWA had a chronic granulomatous keratoconjunctivitis, but otherwise there was no reported evidence of upper or lower respiratory tract disease (de Poister et al., 2011). Clinical signs of this upper respiratory tract disease appear similar to the Mycoplasma agassizii infection of tortoises, but to the author’s knowledge, no PCR or ELISA of swabs, fluids or tissues for mycoplasmas has been performed.

The current treatment regime for bobtails with upper respiratory tract signs is supportive and symptomatic, with hospitalisation in a hot box, rehydration, feeding, and nebulisation forming the cornerstones of care. Treatment also usually involves the administration of the antibiotic enrofloxacin. The rehabilitation success rate is usually very good, approximately 88%, with most bobtails deemed fit for release 8 to 12 weeks after admission (Haight, 2013).

The morbidity associated with the clinical signs appears primarily due to the inability to locate food as eyes may be unable to open, and olfaction is hampered by mucus in the nasal cavity and choana. Ocular disease will also compromise the lizard’s ability to evade predators.

More research is required to investigate the aetiopathogenesis of this disease, of which, haematologic evaluation is one component.
CHAPTER 1: HEALTHY BOBTAIL LIZARDS

1.1 Materials and methods

1.1.1 Lizards

Population
Animal use was in accordance with the Murdoch University Animal Ethics Committee under permit R2447/11. Bobtails were enrolled on a prospective basis. Venepuncture was performed on 52 healthy, adult, captive bobtails, sourced from Armadale Reptile Centre\(^1\), West Australian Reptile Park\(^2\), Caversham Wildlife Park\(^3\), Gary Davies of West Aussie Reptiles\(^4\), and Reptile Trader\(^5\). Bobtails were variably housed in vivariums, indoors and outdoors, and were sampled in summer, between December and March, over two consecutive years (2011/2012, 2012/2013). The diets of the lizards differed at each location. Further details such as age and time in captivity were noted if they were known by the carers.

1 Armadale Reptile Centre, 304-308 South Western Highway, Wungong, Perth, Western Australia 6112, +6189399 6927
2 West Australian Reptile Park, 92 Henley street, Henley Brook, Perth, Western Australia 6055, +6189296 3101
3 Caversham Wildlife Park, Lord street, Caversham, Perth, Western Australia 6055, +6189248 1984
4 West Aussie Reptiles, PO Box 84, Two Rocks, Perth, Western Australia, 6037, +61412 914 537
5 Reptile Trader, 7/ 117 Dixon road, Rockingham, Perth, Western Australia 6168, +6189527 2245

Clinical examination
Lizards were examined and sampled at the location they were kept except the lizards from West Aussie Reptiles which were transported by Gary Davies, by van, to West Australian Reptile Park as per his preference. Any information regarding recent or current medications or illness was noted. A brief physical examination was conducted to determine the state of health of each lizard. The snout to vent length (SVL), tail length and tail thickness was measured and recorded.
**Venepuncture technique**

The technique for venepuncture of the ventral coccygeal vein of *Tiliqua rugosa* has not been previously described. The instruction of the technique for this species was provided by Dr. Tim Oldfield, a veterinarian experienced in the venepuncture of reptiles. It is a modification of the technique described previously for other lizard species (Campbell and Ellis, 2007). The modification was necessary owing to the presence of the large amount of fat stored in the tail, as well as the presence of ventral processes on the coccygeal vertebrae in this species. The technique used in this study is described below.

With the lizard standing on all four feet on a table and facing towards the phlebotomist, the tail was flexed dorsally. The ventral aspect of the tail between 25 and 75% of the length was disinfected using cotton wool soaked in 70% ethanol. A 23G, and ¾, 1, or 1 ½ inch needle (BD, Singapore) on a 2ml syringe containing 80IU of dried heparin (PICO50, Radiometer Medical ApS, Brønshøj, Denmark) was used. The needle was inserted between the scales in the midline of the tail, and then advanced on an angle perpendicular to the tail until the needle contacted the ventral aspect of the coccygeal vertebra, and then withdrawn slightly (Figure 9). If blood was not seen entering the syringe, the needle was redirected. Adult bobtail lizards typically weigh between 300g and 500g; thus a total volume of up to 1.5mls of blood (for a large lizard) was collected and the needle withdrawn. Following gentle agitation in the syringe to ensure dissolution of the heparin, the needle was removed and blood was placed in a 2ml plain plastic tube (Interpath Services, Victoria, Australia). Several blood smears were made immediately using the wedge method and rapidly air-dried as described by Allison and Meinkoth (2007). The blood sample was then placed in a rack inside a chilled Styrofoam box, protected from direct contact with ice and delivered to Murdoch University for analysis within 3 hours of collection.
1.1.2 Laboratory evaluation of blood samples

Complete blood count

Manual blood counts were performed within 6-8 hours of blood collection.

Manual cell counts

Manual cell counts were performed as previously described (Campbell, 1995; Dein et al., 1994; Natt and Herrick, 1952). Samples from 39 lizards were of adequate volume (at least 0.1mls) to perform cell counts. To stain blood cells, a 20µL aliquot of the heparinised blood was placed in a 10ml centrifuge tube (Sarstedt GmbH, Nümbrecht, Germany). Then, 4mls of freshly filtered Natt & Herrick’s solution (Astral Diagnostics, New Jersey, USA) was added to the blood in the centrifuge tube (1:200 dilution). The tube was gently and thoroughly mixed and then left at room temperature for 60 minutes for leukocyte stain uptake. Incubating the cells for 60 minutes improved the staining (pers. obs.) and the differentiation between small lymphocytes and thrombocytes (Robertson and Maxwell, 1990).
After 60 minutes, the centrifuge tube was again gently mixed and using a plain glass capillary tube (Kimble Chase, Tennessee, USA), both sides of an improved Neubauer haemacytometer counting chamber (Weber, Lancing, England) were filled with the solution (Figure 10). The same chamber was used for all cell counts. The chamber was then examined under the Olympus EX41 light microscope (Olympus, Tokyo, Japan).

At 400x magnification, the total number of erythrocytes in the four small corner squares plus the small central square within the central large square was counted. Each small square of the haemacytometer is 0.2mm x 0.2mm and 0.1mm deep (Becton Dickinson VACUTAINER Systems, 1977). Cells that overlapped the top and left borders of the squares were also included. Both sides of the counting chamber were counted and if the counts of the two sides varied by less than 15%, the average cell count was taken. If the CV was greater than 15%, the chamber was reloaded and the cells counted again (Shah et al., 2000; Tomlinson et al., 2013). The red blood cell count was calculated using the formula:

\[ \text{RBC (10}^{12}/\text{L)} = \frac{\text{average number of erythrocytes counted}}{100} \]

Figure 10: The loaded improved Neubauer haemacytometer chamber (arrows indicate leukocytes, arrowheads indicate erythrocytes). (100x magnification, Natt & Herrick’s solution)
At 400x magnification, the total number of leukocytes in the nine large squares was counted. Each large square measures 1mm x 1mm and is 0.1mm deep (Becton Dickinson VACUTAINER Systems, 1977). It was generally possible to differentiate leukocytes from thrombocytes, as thrombocytes were frequently clumped. Due to the clumping, a separate thrombocyte count was not possible. Leukocytes that overlapped the top and left borders of the squares were also included. As for the red blood cell count, the average number of leukocytes over both sides of the chamber was taken where there was a difference of less than 15% between them. The white blood cell count was calculated using the formula:

\[
\text{WBC (10}^9/\text{L)} = \frac{((\text{average number of leukocytes counted} + 10\% \text{ of total leukocytes}) \times 2)}{10}
\]

All counts were performed at a rapid enough rate to ensure that drying of the chamber was not encountered.

As manual methods are known to be less accurate than automated methods, a repeatability study was performed to validate the manual cell counts. With one sample, both sides of the haemacytometer chamber were loaded ten times, and the number of red blood cells and white blood cells were counted as per the methods described above.

**Packed cell volume and total plasma protein**

The packed cell volume and total plasma protein were performed using microhaematocrit tubes with the widely used methods of microhaematocrit centrifugation and refractometry respectively. Briefly, a plain capillary tube (Kimble Chase, Tennessee, USA) was filled with the heparinised blood and heat sealed. The tube was then spun in the Heraeus Biofuge haemo microhaematocrit centrifuge (Kendro Laboratory Products, Hanau, Germany) for 5 minutes at 16,060g. The packed cell volume was measured using a graduated chart. The colour of the plasma was noted, and then the capillary tube was broken at the interface of the buffy coat and the plasma towards the top of the plasma, and the separated plasma was
allowed to fill the window of the hand-held AO TS Meter refractometer (Reichert Goldberg, New York, USA) by capillary action. The total plasma protein was visualised and recorded.

**Haemoglobin concentration**

Following all other analyses, a minimum volume of 200µL of heparinised blood was submitted to Vetpath Laboratory Services. Sufficient total blood volume was collected from 32 lizards. Blood was stored at 4°C and analysed by Vetpath 6-24 hours following collection. The haemoglobin concentration was measured by a CELL-DYN 3700 haematology analyser (Abbott Laboratories, Illinois, USA) using the Resistant RBC mode which places the blood in contact with a lysing sheath reagent for 15 seconds longer than the regular RBC mode. This analyser has been validated for reptile haematology (Vap et al., 2012). The CELL-DYN 3700 measures haemoglobin using a modified hemiglobin-hydroxylamine method. The lysing reagent converts the haemoglobin to a single chromagen, the hemiglobin-hydroxylamine complex. A LED light source emits light which passes through the lysed blood and the light is detected by a photodetector with an interference filter for 540nm. This is the wavelength at which the hemiglobin-hydroxylamine complex is measurable, and the resultant amount of light detected by the photodetector reflects the number of complexes present in the sample. As per the manufacturer recommendations, the haemoglobin was then manually corrected by subtracting a haemoglobin correction value. The required haemoglobin correction value was obtained from a chart provided by the manufacturer, which varies with the calculated red blood cell count of the sample. This correction removes interference by the erythrocyte nuclei.

6 Vetpath Laboratory Services, PO Box 18, Belmont, Perth, Western Australia 6984, +6189259 3666

**Blood smear examination**

Immediately following collection, blood smears were made from the heparinised blood using the wedge method (Allison and Meinkoth, 2007). The smears were air-dried and stained using the automated Hematek® Slide Stainer (Ames Company, Indiana, USA) with Hematek® Stain Pak Wright-Giemsa stain (Siemens Healthcare, Victoria, Australia) within 4
hours of smear preparation. Rapid staining (Amber Scientific, Midvale, Australia) was performed on six smears, also within 4 hours of smear preparation.

Vetpath Laboratory Services prepared smears on refrigerated blood stored at 4°C for 6-24 hours. Blood smears from four lizards were stained with Leishman’s stain: a combination of May-Grunwald Farbstoff powder (Schmid GmbH + Co, Köngen, Germany) and Leishman’s stain powder (Hurst Scientific, Perth, Australia). Ten new methylene blue stains (Chem-Supply, Gillman, Australia) were also prepared.

All Wright’s-Giemsa smears were examined at 100x, 400x and 1000x magnification on the Olympus EX41 microscope (Olympus Corporation, Tokyo, Japan) and the morphology and staining characteristics were described for each cell. A Nikon Eclipse 80i microscope with a Nikon DS Camera Control Unit DS-L2 and associated DS-L2 software (Nikon, Tokyo, Japan) was used to measure the diameter of five cells of each cell type under oil immersion (1000x magnification) and the mean was used where the morphology was similar, or the range for more pleomorphic cell types. Measurements were made over 3-5 smears from different bobtails. All images were also taken by the same unit.

In addition to Romanowsky and Romanowsky-type stains, cytochemical staining was also performed on some smears. Peroxidase (benzidine), toluidine blue, alcian blue, periodic acid-Schiff and oil red O stains were performed on eight, four, three, three and two smears, respectively. All methods were routine and performed by the histology laboratory at Murdoch University according to the standard protocols for the laboratory. Smears were examined under 100x, 400x and 1000x magnifications and the staining characteristics were noted for each cell type.

**Erythrocytes**

The degree of polychromasia was given as the number of polychromatophils per ten 1000x fields in the monolayer of the blood smear, then classified as nil to marked, as per the criteria defined by Campbell (2004b). Briefly, slight polychromasia is defined as 2 to 10 polychromatophils per 1000x field. Mild polychromasia is 11 to 14 polychromatophils per 1000x field. Moderate polychromasia is 15 to 30 polychromatophils per 1000x field. Marked
polychromasia is greater than 30 polychromatophils per 1000x field. This classification scheme posed a problem in that the blood cell distributions of the smears were not even and uniform. To rectify this, on several well-made and regularly distributed smears, the mean number of erythrocytes per 1000x field was counted in the monolayer. This number was estimated to be 100 erythrocytes per 1000x field. Thus, on all smears, the mean number of polychromatophils per 100 red blood cells was counted instead, from a total of 1000 erythrocytes. This enabled the degree of polychromasia to be given as a percentage and to be classified as above.

The severity of poikilocytosis in the monolayer was also assessed as slight to marked, again as per Campbell (2004b). Slight poikilocytosis was noted if 5 to 10 poikilocytes were seen per 1000x field. Mild poikilocytosis if 11 to 20 poikilocytes were seen per 1000x field. Moderate poikilocytosis was 21 to 50 poikilocytes per 1000x field. Marked poikilocytosis was noted if there were more than 50 poikilocytes per 1000x field.

The number of haemogregarine parasites per 10,000 red blood cells (or approximately ten 1000x fields) was also recorded.

**Leukocytes**

The leukocytes were classified and a 200 cell leukocyte differential was performed on a Wright’s-Giemsa smear from each lizard at 400x magnification. The zig-zag or cross-sectional method of counting was used within the monolayer of each smear (MacGregor et al., 1940).

In the heterophils, any toxic changes were noted and classified as 1+, 2+, 3+, or 4+ depending upon the degree of toxicity as per Campbell (2004b). A 1+ toxic heterophil has increased cytoplasmic basophilia. A 2+ toxic heterophil has increased cytoplasmic basophilia, mildly abnormal granulation such as partial degranulation, coalescing granules, or abnormal appearing granules, or vacuolation. A 3+ toxic heterophil will show changes that are more severe than the 2+ toxicity and the nucleus may show slight karyorhexis or karyolysis. A 4+ toxic heterophil has both marked nuclear and cytoplasmic changes.
Immunoreactive lymphocytes are characterised by enhanced cytoplasmic basophilia and/or a perinuclear clearing. In addition to counting them as lymphocytes in the differential, immunoreactive lymphocytes were counted separately and expressed as number per 100 white blood cells (Raskin, 2000; Stacy et al., 2011).

**White blood cell count smear estimate**

In addition to the haemacytometer manual counting method, white blood cell counts were also estimated from the blood smears of 45 lizards. Smear estimates are less accurate than manual haemacytometer counts, but have been suggested as a useful quality control method to check the haemacytometer count (Harr et al., 2001; Strik et al., 2007).

Two slightly different, previously reported white cell estimate methods were used. To the author’s knowledge, these are the only white blood cell count estimates suggested for use on reptile blood smears. Both methods appear similar but both were used in this study to assess whether one was more accurate than the other compared to the manual count.

Method 1 involved calculating the mean number of leukocytes per microscopic field, then multiplying the result by the objective power squared (Harr et al., 2001). The mean was taken over ten fields. The 400x magnification was used for all counts as this was the magnification that enabled correct identification of leukocytes, particularly differentiation of lymphocytes from thrombocytes, and polychromatophils from lymphocytes. The result was an estimate of the number of white blood cells per microlitre, which was converted to $10^9$/L by dividing by 1000.

Method 2 is a modification of the first method, similar to one reported for use in birds (Campbell and Coles, 1986). Method 2 uses the magnification at which there are 5-10 leukocytes per field. This varied between the smears from 200x to 1000x magnification. The average number of cells per field was calculated over ten fields and the mean was used. This result was multiplied by the objective power squared to give an estimate of the number of white blood cells per microlitre (Strik et al., 2007). This was then converted to $10^9$/L.
**Thrombocytes**

The clumping of thrombocytes precluded counting. Morphology and size assessment of individual cells was performed.

**Plasma biochemistry**

Following haematological analysis, there was a sufficient volume of heparinised plasma remaining from eight healthy bobtails to measure a small panel of biochemistry analytes. The blood was centrifuged at 1500g for 5 minutes by the Thermo Scientific Jouan cR3i multifunction centrifuge (Jouan, Saint-Herblain, France), and the plasma was separated and stored at -30°C for 9 months. Plasma total protein, albumin, inorganic phosphate, total calcium, glucose and uric acid concentrations were measured with the Rx Daytona clinical chemistry analyser and Randox reagents (Randox, Antrim, United Kingdom). The methods used for chemistry analysis by the Daytona were: the biuret method for total protein, bromocresol green for albumin, the UV method (ammonium molybdenate; 340nm) for inorganic phosphate, the colorimetric method for total calcium (arsenazo III; 660nm), the hexokinase method for glucose, and the enzymatic colorimetric method (uricase and peroxidase; 546nm) for uric acid.

1.1.3 Transmission electron microscopy

From the blood samples of healthy bobtails (Healthy 1, 42, 48, 49), a 40µL aliquot of heparinised whole blood was immediately pipetted and placed in a chilled 1.5ml Eppendorf tube (Sarstedt GmbH, Nümbrecht, Germany) following blood collection. This was followed by the addition of 80µL of chilled 5% glutaraldehyde to each aliquot of blood and then the Eppendorf tubes were placed in a chilled Styrofoam container and protected from contact with ice. Upon returning to Murdoch University, within 3-4 hours of blood collection, the fixed blood samples were placed in a refrigerator at 4°C.
**Fixation, dehydration and embedding of the cell pellet**

The Eppendorf tubes which contained the fixed, heparinised whole blood were centrifuged at 1500g for 5 minutes by the Thermo Scientific Jouan cR3i multifunction centrifuge (Jouan, Saint-Herblain, France) to form cell pellets. The plasma was removed from each sample and discarded. The cell pellets were carefully lifted from the bottom of the tube and post-fixed with 40µL of Dalton’s chrome osmic acid to cover the pellet. The cell pellet was left to fix for 1.5 hours and agitated intermittently to increase the exposure of the sample.

The cell pellet was then processed according to the routine protocol for all tissues at Murdoch University. The osmic acid was discarded and each cell pellet was dehydrated though graded alcohols: four washes with 70% ethanol, then 90% ethanol for 5 minutes, 95% ethanol for 5 minutes and three changes of absolute (100%) ethanol over 10 minutes. The ethanol was discarded and the fixed cell pellet was washed in two changes of propylene oxide over 10 minutes. The cell pellet was then covered by a 60:40 solution of propylene oxide/epoxy resin and kept for 1 hour at 4°C in a refrigerator. The propylene oxide/epoxy resin was discarded and the pellet was covered with epoxy resin and left overnight on a rotator, with the lid off the Eppendorf tube. The following day, the epoxy resin was removed, fresh resin was added - again to cover the pellet - and the tube was baked in the oven at 60°C for 24 hours to embed the pellet.

**Sectioning and microscopic examination**

To ensure the samples were adequate for electron microscopy, each embedded cell pellet was cut into 1µm thick sections using a glass knife on the Om U3 Ultramicrotome (Reichert, Vienna, Austria), expanded with chloroform, warmed and fixed on a glass slide (Lewis et al., 1974; Reid, 1975). The sections were stained with toluidine blue and examined under light microscopy.

Multiple ultrathin sections, 90nm thick (gold interference), were cut using a glass knife on the Reichert Ultracut E ultramicrotome (Reichert-Jung, Vienna, Austria) and mounted on copper grids. The grids were stained with refrigerated uranyl acetate and then lead citrate. Grids were examined using the Philips CM100 Bio transmission electron microscope at 80kV.
(Philips, Eindhoven, The Netherlands). Photomicrographs were taken with an exposure time of 2.07 to 2.61 seconds.

### 1.1.4 Statistical analysis

Significance was set at P<0.05 for all analyses performed.

**Statistical analysis of the population**

The mean and standard deviation of the snout-to-vent length and tail thickness of the healthy bobtails was calculated using Microsoft® Excel® 2010 (Microsoft Corporation, Redmond, USA).

A bivariate correlation study was performed to compare the volume of blood obtained to the tail thickness using IBM SPSS Statistics Version 19 (IBM Corporation, Somers, USA).

**Statistical analysis of the repeatability study**

For the validation of the manual cell counts, the coefficient of variation (CV) was calculated for the red blood cell count and white blood cell count: CV= SD/mean x 100 (Chesher, 2009). As another measure of repeatability, a Bland-Altman plot was constructed using IBM SPSS Statistics Version 19 (IBM, Somers, USA) with upper and lower bounds (two standard deviations). Further, the correlation between the counts for each side of the chamber was calculated using IBM SPSS Statistics Version 19 (IBM, Somers, USA).

**Statistical analysis of the laboratory data**

Reference intervals were calculated for PCV, total plasma protein, RBC count, polychromasia, WBC count, and the leukocyte differential counts using the Reference Value Advisor v2.1 macro for Microsoft® Excel® (National Veterinary School of Toulouse, Toulouse, France) (Geffré et al., 2011). The Reference Value Advisor software produced tables,
histograms, dot plots and Q-Q plots of the data. It identified outliers using two methods: Dixon’s outlier range statistic and Horn’s algorithm using Tukey’s interquartile fences. The Anderson-Darling test was used to assess the goodness-of-fit to a Gaussian distribution (normality). It calculated reference intervals on both standard and robust, untransformed and Box-Cox transformed data. Robust methods are recommended for between 40 and 120 samples. Where the sample size was greater than 40, it also calculated the intervals using a nonparametric bootstrap method. Finally, 90% confidence intervals for the upper and lower limits of the reference intervals were calculated. The size of the 90% confidence intervals indicates the degree of uncertainty or imprecision in the interval (Braun et al., 2013; Friedrichs et al., 2011; Geffré et al., 2011).

For each parameter, identified Dixon and Tukey’s outliers were removed and the reference intervals were recalculated. Tukey suspect outliers were generally left in as per the CLSI-IFCC C28-A3 guideline (Clinical and Laboratory Standards Institute, 2008). Between three and five sets of reference intervals were calculated by the software, depending on the sample size and distribution of the data. The most appropriate reference interval to use for each parameter was determined by assessing whether the data was Gaussian using an Anderson-Darling test (P>0.05), then assessing the width of the confidence intervals. The CLSI and QALS Committee recommend that the width of the 90% confidence interval be less than 0.2 times the width of the reference interval for the upper and lower limits (Clinical and Laboratory Standards Institute, 2008; Friedrichs et al., 2011). The wider the interval, the greater the degree of uncertainty. The collection of additional samples is recommended if the width is greater than 0.2 (Friedrichs et al., 2011). It is recognised that this is not always possible with smaller data sets, particularly those with less than 55 individuals (Braun et al., 2013). Thus the reference intervals with the narrowest confidence intervals were selected.

Once the reference intervals were constructed, further analysis of the healthy bobtail data was performed using IBM SPSS Statistics Version 19 (IBM, Somers, USA). A series of correlation analyses were performed for all analytes. The strength of correlations was categorised according to previously published guidelines (Asuero et al., 2006). Briefly r>0.9 was considered very high, r=0.7-0.89 was high, r=0.5-0.69 was moderate, and r=0.3-0.49 was considered of low correlation.
The effect of low blood volume on PCV, Hb, RBC count and WBC count was assessed using independent t-tests, or a Mann-Whitney U test (WBC).

The effect of the presence of haemogregarines parasites on PCV, Hb, RBC count and WBC count was evaluated using independent t-tests, or a Mann-Whitney U test (WBC).

A Pearson’s correlation analysis was performed between PCV and the polychromatophils count.

Pairwise comparisons were made between the manual WBC count, WBC estimate Method 1 and 2, with a Bonferroni adjustment.

Independent t-tests or Mann-Whitney U tests (WBC count, basophils, haemogregarines count, reactive lymphocyte count and polychromatophils count) were performed to compare all results from bobtails housed indoors to those housed outdoors. The mean or median differences were assessed.

The PCV, Hb, RBC count and WBC count results from the Canfield and Shea (1988) study were compared to the results from this study using independent t-tests.

A Pearson’s correlation analysis between total plasma protein as measured by refractometry, and total protein as measured by the biuret method was performed using Microsoft® Excel® 2010 (Microsoft Corporation, Redmond, USA).
1.2 Results

1.2.1 Bobtail morphometrics

A total of 52 healthy, captive, adult lizards were sourced from wildlife parks and private collections in Perth. The snout to vent length and tail thickness, just caudal to the pelvis, was measured for each lizard. Normality was confirmed by the Anderson-Darling test. The snout to vent length ranged from 225-305mm, with a mean of 277mm (SD 17). The tail thickness varied from 21-38mm, with a mean of 30mm (SD 3.09) (Table 1).

1.2.2 Volume of blood collected

The volume of blood collected was compared to the tail thickness to assess whether there was a correlation. Of the 52 healthy lizards included in the study, no blood could be obtained from six individuals (Table 1). From an additional six lizards, only a drop or so of blood was collected, sufficient only to prepare a smear. From those bobtails where a good flow of blood was obtained, the maximum volume of blood collected was 1-1.5mls. There was no correlation between tail thickness and volume of blood collected (Pearson’s correlation coefficient r=0.076).

Depending on the volume obtained, at least one blood smear was made (n=46), a PCV and total plasma protein was measured (n=40), red blood cell and white blood cell counts were performed (n=39), and haemoglobin was measured (n=32). Analyses were prioritised in that order.
Table 1: Healthy lizard data. Highlighted in yellow are snout to vent lengths which were greater than two standard deviations outside the mean. Table continued overleaf.

<table>
<thead>
<tr>
<th>#</th>
<th>Source</th>
<th>Housing</th>
<th>Date collected</th>
<th>S-VL (mm)</th>
<th>Tail thickness (mm)</th>
<th>Time in captivity</th>
<th>Sloughing</th>
<th>Blood volume collected (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Armadale Reptile Centre</td>
<td>1</td>
<td>16/01/2012</td>
<td>290</td>
<td>29</td>
<td>Unknown</td>
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</tr>
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<td>23/01/2012</td>
<td>240</td>
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<td>0.2</td>
</tr>
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<td>5</td>
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<td>23/01/2012</td>
<td>250</td>
<td>28</td>
<td>Lifelong (approx 5 years)</td>
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<td>0.7</td>
</tr>
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<td>23/01/2012</td>
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<td>Lifelong (approx 5 years)</td>
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<td>0.5</td>
</tr>
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<td>Lifelong (approx 5 years)</td>
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<td>1.0</td>
</tr>
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<td>Lifelong (approx 5 years)</td>
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<td>0.7</td>
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<td>1/02/2012</td>
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<td>0.7</td>
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<td>32</td>
<td>3 years; in captivity since approx 6 months old</td>
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<td>0.03</td>
</tr>
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</tr>
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<td>0.03</td>
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<td>Unknown</td>
<td>0</td>
<td>0.5</td>
</tr>
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<td>1.0</td>
</tr>
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<td>260</td>
<td>28</td>
<td>Unknown</td>
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Table 1 continued: Healthy lizard data.

<table>
<thead>
<tr>
<th>#</th>
<th>Source</th>
<th>Housing</th>
<th>Date collected</th>
<th>S-VL (mm)</th>
<th>Tail thickness (mm)</th>
<th>Time in captivity</th>
<th>Sloughing</th>
<th>Blood volume collected (ml)</th>
</tr>
</thead>
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<td>16/01/2013</td>
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<td>28</td>
<td>A few weeks- wild caught in backyard</td>
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</tr>
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<td>0</td>
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</tr>
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</tr>
<tr>
<td>42</td>
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<td>265</td>
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<td>Unknown</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>43</td>
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<td>270</td>
<td>29</td>
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<td>0</td>
<td>1</td>
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<tr>
<td>44</td>
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</tr>
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<td>0</td>
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<td>0</td>
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<td>49</td>
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<td>0</td>
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<td>270</td>
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<tr>
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<td>0.01</td>
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<tr>
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<td>280</td>
<td>29</td>
<td>Unknown</td>
<td>0</td>
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</tr>
</tbody>
</table>

Mean 277 0.5
SD 42 0.4
Median 280 0.5
Range 225-305 0-1.5
Bobtails were sourced from five different locations. They were variably housed indoors or outdoors. For the most part, the length of time in captivity was not known by the keepers. Six bobtails were partially sloughing (ecdysis) at the time of collection.

**1.2.3 Complete blood count**

**Repeatability study**

To validate the manual cell count methods, a repeatability study was performed on one sample. A total of ten counts in duplicate, were performed (Table 2).

<table>
<thead>
<tr>
<th>Chamber number</th>
<th>Number of cells counted</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RBC 1</td>
<td>RBC 2</td>
<td>WBC 1</td>
</tr>
<tr>
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<td>33</td>
<td>34</td>
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</tr>
<tr>
<td>2</td>
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<td>39</td>
<td>13</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>Mean</th>
<th>Standard deviation</th>
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</thead>
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<td>Number of cells counted</td>
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<td>3.83</td>
</tr>
<tr>
<td>WBC 1</td>
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<td>16.7</td>
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</tr>
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<td>WBC 2</td>
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</table>

The coefficient of variation (CV) for the red blood cell count was 10%. The CV for the white blood cell count was 20%. For the red blood cell counts, there was a moderate positive correlation \( r=0.500 \) between the counts obtained from either side of the chamber (RBC1 and RBC2). There was no correlation \( r=-0.147 \) between the counts either side of the chamber for the white blood cell count (WBC1 and WBC2). On a Bland-Altman plot, all red blood cell data points fell within the upper and lower bounds i.e., two standard deviations. There were two data points for the white blood cell count which fell outside these bounds (Figure 11).
Figure 11: Bland-Altman plot of the difference between chamber sides over ten cell counts of the same sample (Diff= difference, LB=lower bound, UB=upper bound). Microsoft® Excel® graph (Microsoft Corporation, Redmond, USA).

**Reference intervals**

Following removal of the outliers identified by Dixon or Tukey methods, reference intervals were calculated for the following data: PCV, total plasma protein, RBC count, haemoglobin, MCV, MCH, MCHC, percentage polychromatophils, WBC count, percentage heterophils, eosinophils, basophils, monocytes, lymphocytes, and smear white blood cell estimates (Tables 3-5).

Table 3: Summary data of the erythron and total plasma protein of healthy bobtail lizards.

<table>
<thead>
<tr>
<th>Test</th>
<th>PCV</th>
<th>Hb</th>
<th>RBC</th>
<th>MCV</th>
<th>MCH</th>
<th>MCHC</th>
<th>Polychromatophils</th>
<th>Total plasma protein</th>
</tr>
</thead>
<tbody>
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<td>pg</td>
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Table 4: Summary data of the leukon of healthy bobtail lizards.

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<th>WBC</th>
<th>Heterophils</th>
<th>Monocytes</th>
<th>Lymphocytes</th>
<th>Eosinophils</th>
<th>Basophils</th>
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<td>%</td>
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Table 5: Summary data of the white blood cell count smear estimates of healthy bobtail lizards.

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<tr>
<th>Test</th>
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<th>WBC smear estimate Method 2</th>
<th>Manual haemacytometer WBC</th>
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<td>x10^9/L</td>
<td>x10^9/L</td>
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<td>Mean</td>
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<td>11.15</td>
<td>9.49</td>
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<td>Median</td>
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<td>11.04</td>
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<td>SD</td>
<td>5.39</td>
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<td>2.48</td>
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<td>30.96</td>
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</table>

The following results and graphs are derived from the Reference Value Advisor freeware v2.1 (National Veterinary School of Toulouse, Toulouse, France) (Figures 12-27). The dot plots have been reproduced following exclusion of outliers to illustrate the range of the data and illustrate any suspect outliers (Tukey) which have been retained. The histograms have also been reproduced to illustrate the distribution and symmetry of the data. The presentation of the reference limits and 90% confidence intervals produced by the freeware macro has been modified to improve the clarity of the graphs. The untransformed histograms are included where the reference intervals were obtained by the untransformed robust parametric method, or the nonparametric bootstrap method where data were non-Gaussian. In analytes where the reference intervals were narrowest following Box-Cox transformation, both the untransformed and transformed histograms are presented. The
untransformed histograms have been included to provide a better indication of the distribution of the data prior to transformation.

Figure 12: Dot plot and histogram of the packed cell volume data.

No outliers were identified for PCV. The data was Gaussian (Anderson-Darling P=0.40). The untransformed, robust 90% confidence intervals were the most narrow and thus were selected.
Figure 13: Dot plot and histogram of the total plasma protein (refractometry) data.

For the total plasma protein, one outlier was identified and removed. The data was Gaussian (Anderson-Darling $P=0.31$). The untransformed, robust 90% confidence intervals were the most narrow and thus were selected.
Figure 14: Dot plot and histograms (untransformed, top, and Box-Cox transformed, bottom) of the haemoglobin data.

No outliers for haemoglobin concentration were identified. The narrowest 90% confidence intervals were the Box-Cox transformed, robust intervals; hence they were selected. Both the untransformed and Box-Cox transformed data were Gaussian (Anderson-Darling P=0.72 and P=0.84 respectively).
Figure 15: Dot plot and histograms (untransformed, top, and Box-Cox transformed, bottom) of the red blood cell count data.

One outlier for the red blood cell count was identified and removed. The robust Box-Cox-transformed 90% confidence intervals were the narrowest and thus they were selected. Both data were Gaussian (Anderson-Darling P=0.55 and P=0.57 respectively).
Figure 16: Dot plot and histograms (untransformed, top, and Box-Cox transformed, bottom) of the mean corpuscular volume data.

For MCV, no outliers were identified. Overall, the robust Box-Cox-transformed 90% confidence intervals were the narrowest and were selected. Both data were Gaussian (Anderson-Darling P=0.08 and P=0.32 respectively).
Figure 17: Dot plot and histograms (untransformed, top, and Box-Cox transformed, bottom) of the mean corpuscular haemoglobin data.

There were no outliers for MCH. The 90% confidence intervals for the robust Box-Cox transformed data were overall the narrowest and selected. Both data were Gaussian (Anderson-Darling P=0.20 and P=0.52 respectively).
Figure 18: Dot plot and histograms (untransformed, top, and Box-Cox transformed, bottom) of the mean corpuscular haemoglobin concentration data. x= suspect Tukey outliers

For the MCHC, three outliers were identified and removed. Two suspect outliers were retained. Only the Box-Cox transformed data was Gaussian (Anderson-Darling P=0.24; untransformed data P=0.03) so the robust Box-Cox transformed reference intervals were used.
Figure 19: Dot plot and histogram of the polychromatophils data. x= suspect Tukey outliers

One outlier was removed and the three suspect outliers were retained with the polychromatophils data. The data was not Gaussian (Anderson-Darling P=0.00) and had a left-skew. The sample size was 45 so the non-parametric reference intervals were used.
Figure 20: Dot plot and histograms (untransformed, top, and Box-Cox transformed, bottom) of the white blood cell count data.

There were no identified outliers in the white blood cell count data. Only the Box-Cox transformed data was Gaussian (Anderson-Darling P=0.06; untransformed data P=0.00). Thus the Box-Cox transformed reference intervals were used.
Figure 21: Dot plot and histogram of the heterophils data.

No outliers were identified in the heterophils data. The data was Gaussian (Anderson-Darling $P=0.94$). The reference intervals for the untransformed robust data were selected as they were the narrowest.
One outlier was identified and removed from the monocytes data. The narrowest reference intervals were those generated from the Box-Cox transformed data so they were selected. Both data were Gaussian (Anderson-Darling P=0.87).
In the lymphocytes data, two outliers were identified and removed. The narrowest reference intervals were the Box-Cox transformed data, thus they were selected. Both data were Gaussian (Anderson-Darling $P=0.83$ and 0.89 respectively).
Figure 24: Dot plot and histograms (untransformed, top, and Box-Cox transformed, bottom) of the eosinophils data. $\times$ = suspect Tukey outliers

For the eosinophils data, no strong outliers were identified, and one suspect outlier was retained. Only the Box-Cox transformed data was Gaussian (Anderson-Darling $P=0.89$; untransformed data $P=0.00$) thus the Box-Cox transformed reference intervals were used.
For the basophils data, no strong outliers were detected but the two suspect outliers were retained. The data was not Gaussian (Anderson-Darling P=0.00). The sample size was large enough to calculate nonparametric reference intervals with the bootstrap method, and those intervals were used.

Figure 25: Dot plot and histogram of the basophils data. ×= suspect Tukey outliers
Figure 26: Dot plot and histograms (untransformed, top, and Box-Cox transformed, bottom) of the white blood cell count smear estimate Method 1 data. x = suspect Tukey outliers

With the white blood cell count smear estimate Method 1, two outliers were identified and removed. Four suspect outliers were retained. Only the Box-Cox transformed data was Gaussian (Anderson-Darling P=0.36; untransformed data P=0.00). Therefore, the Box-Cox transformed reference intervals were used.
Figure 27: Dot plot and histogram of the white blood cell count smear estimate Method 2 data. $\times$= suspect Tukey outliers

Five outliers were removed and the three suspect outliers were retained for the data from white blood cell count smear estimate Method 2. The data was not Gaussian (Anderson-Darling P=0.00). Nonparametric reference intervals were used as the sample size was large enough to calculate them (using the bootstrap method).

In summary, untransformed, robust reference intervals were produced for PCV, total plasma protein, and percentage heterophils. Box-Cox, robust reference intervals were selected for haemoglobin concentration, red blood cell count, MCV, MCH, MCHC, white blood cell count, percentage monocytes, lymphocytes, and eosinophils, as well as white blood cell count smear estimate method 1. Non-Gaussian distributions necessitated that nonparametric reference intervals were calculated for percentage basophils, degree of polychromasia and white blood cell count smear estimate method 2.
Correlations and factors affecting the complete blood count

IBM SPSS Statistics software (IBM, Redmond, USA) was used to analyse the healthy bobtail data. A Shapiro-Wilk test was used to confirm normality for each analyte. A series of correlations were performed between all analytes (Table 6 and 7).
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<th>PCV</th>
<th>RBC</th>
<th>WBC</th>
<th>Hb</th>
<th>Monocytes</th>
<th>Heterophils</th>
<th>Lymphocytes</th>
<th>Eosinophils</th>
<th>Basophils</th>
<th>Parasites per 10000 rbc</th>
<th>Reactive lymphocytes per 100 wbc</th>
<th>Polychromatophils per 100 rbc</th>
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<td><strong>Basophils</strong></td>
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<tr>
<td>Pearson Correlation</td>
<td>-0.370**</td>
<td>0.346**</td>
<td>0.067</td>
<td>0.116</td>
<td>0.208</td>
<td>0.147</td>
<td>-0.107</td>
<td>0.171</td>
<td>0.084</td>
<td>0.220</td>
<td>0.141</td>
<td>0.007</td>
<td>0.396**</td>
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<tr>
<td>Sig. (2-tailed)</td>
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<tr>
<td><strong>Parasites</strong></td>
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<tr>
<td>Pearson Correlation</td>
<td>-0.092</td>
<td>0.129</td>
<td>0.002</td>
<td>-0.003</td>
<td>0.022</td>
<td>1</td>
<td>0.393**</td>
<td>0.037</td>
<td>0.076</td>
<td>0.816</td>
<td>0.147</td>
<td>0.287</td>
<td>0.066</td>
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<td>Sig. (2-tailed)</td>
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<td><strong>Reactive lymphocytes</strong></td>
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<tr>
<td>Pearson Correlation</td>
<td>-0.370**</td>
<td>0.346**</td>
<td>0.067</td>
<td>0.116</td>
<td>0.208</td>
<td>0.147</td>
<td>-0.107</td>
<td>0.171</td>
<td>0.084</td>
<td>0.220</td>
<td>0.141</td>
<td>0.007</td>
<td>0.396**</td>
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<td>Sig. (2-tailed)</td>
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<td><strong>Polychromatophils</strong></td>
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<tr>
<td>Pearson Correlation</td>
<td>-0.092</td>
<td>0.129</td>
<td>0.002</td>
<td>-0.003</td>
<td>0.022</td>
<td>1</td>
<td>0.393**</td>
<td>0.037</td>
<td>0.076</td>
<td>0.816</td>
<td>0.147</td>
<td>0.287</td>
<td>0.066</td>
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<tr>
<td>Sig. (2-tailed)</td>
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</tbody>
</table>
Table 7: Summary data of all the significant (P<0.05) correlations of haematology data.

<table>
<thead>
<tr>
<th>Type of correlation</th>
<th>High correlation r=0.7-0.89</th>
<th>Moderate correlation r=0.5-0.69</th>
<th>Low correlation r=0.3-0.49</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>PCV + Hb</td>
<td>RBC + TP</td>
<td>PCV + TP, Hb + RBC, PCV + RBC, Hb + Baso, Hb + TP, PCV + Baso, Parasites + Poly</td>
</tr>
<tr>
<td>-</td>
<td>Hetero + Lymph</td>
<td></td>
<td>Hb + Eos, Hetero + Eos, PCV + WBC, WBC + Lymph, TP + Parasites, PCV + Parasites, Hetero + Baso</td>
</tr>
</tbody>
</table>

There were many significant correlations within the complete blood count data, summarised in Table 7. The majority of the correlations were low. There were no very high correlations (r>0.9).

The effect of low collected blood volume on PCV, RBC counts, haemoglobin and WBC counts was investigated using independent t-tests or Mann-Whitney U test (WBC). Data was divided into greater than or equal to 1ml of blood collected, and less than 1ml collected; then 0.5mls of greater of blood collected, and less than 0.5mls (Table 8).

Table 8: Effect of blood volume collected on complete blood count analytes (significance P<0.05) (*Mann-Whitney U test).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>≥1ml vs &lt;1ml</th>
<th>≥0.5mls vs &lt;0.5mls</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV</td>
<td>0.760</td>
<td>0.935</td>
</tr>
<tr>
<td>RBC</td>
<td>0.794</td>
<td>0.393</td>
</tr>
<tr>
<td>Hb</td>
<td>0.966</td>
<td>0.996</td>
</tr>
<tr>
<td>WBC*</td>
<td>0.679</td>
<td>0.326</td>
</tr>
</tbody>
</table>

There were no significant differences found with PCV, red blood cell count, haemoglobin concentration or white blood cell count at a higher or lower blood volume.

A correlation analysis was performed to compare PCV with the degree of polychromasia (%). This correlation is illustrated diagrammatically (Figure 28).
There was significant (P=0.041) yet low and negative correlation between PCV and polychromasia (Pearson’s correlation $r=-0.329$).

The data from bobtails housed outdoors (without any lighting or heating) was compared to those housed indoors using independent t-tests, or Mann-Whitney U tests for non-Gaussian analytes (WBC count, basophils, parasites, reactive lymphocytes, polychromatophils) (Table 9).

Table 9: The effect of housing on haematology data.

<table>
<thead>
<tr>
<th>Number of healthy bobtails (n)</th>
<th>Housing</th>
<th>PCV</th>
<th>Haemoglobin</th>
<th>RBC</th>
<th>WBC</th>
<th>Polychromasia</th>
<th>Haemogregarines</th>
<th>Total plasma protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indoor</td>
<td>19</td>
<td>14</td>
<td>17</td>
<td>18</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Outdoor</td>
<td>21</td>
<td>18</td>
<td>21</td>
<td>21</td>
<td>25</td>
<td>26</td>
<td>26</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>32</td>
<td>38</td>
<td>39</td>
<td>45</td>
<td>46</td>
<td>46</td>
<td>39</td>
</tr>
<tr>
<td>Significance</td>
<td>0.008</td>
<td>0.077</td>
<td>0.297</td>
<td>0.822</td>
<td>0.006</td>
<td>0.129</td>
<td>0.284</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of healthy bobtails (n)</th>
<th>Housing</th>
<th>Heterophils</th>
<th>Monocytes</th>
<th>Lymphocytes</th>
<th>Eosinophils</th>
<th>Basophils</th>
<th>Reactive lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indoor</td>
<td>20</td>
<td>19</td>
<td>18</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Outdoor</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td>46</td>
<td>45</td>
<td>44</td>
<td>46</td>
<td>46</td>
<td>46</td>
<td>46</td>
</tr>
<tr>
<td>Significance</td>
<td>0.023</td>
<td>0.559</td>
<td>0.409</td>
<td>0.227</td>
<td>0.010</td>
<td>0.439</td>
<td></td>
</tr>
</tbody>
</table>
In the outdoor bobtails, the PCV was significantly lower (P=0.008) with a mean difference of 0.06 L/L. The basophil count was also significantly lower (P=0.010) with a median difference of 3.5%. The heterophil count was significantly higher (P=0.023) with a mean difference of 8.4%. The polychromatophils were also significantly higher (P=0.006) with a median difference of 1.1%.

The smears from 13 bobtails contained haemogregarine parasites (Table 10). The PCV, RBC counts, haemoglobin and WBC counts of bobtails with and without haemogregarine parasites were compared using independent t-tests, or a Mann-Whitney U test (WBC).

Table 10: Number of bobtails with and without haemogregarine parasites.

<table>
<thead>
<tr>
<th>Smear</th>
<th>Number of healthy bobtails (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCV</td>
</tr>
<tr>
<td>Haemogregarines detected</td>
<td>13</td>
</tr>
<tr>
<td>No haemogregarines detected</td>
<td>34</td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
</tr>
</tbody>
</table>

The PCV was significantly lower in the haemogregarine-infected bobtails (P=0.041). The mean difference was 0.04 L/L. There was no difference between infected and non-infected bobtails for the RBC or WBC count (P=0.101 and P=0.512 respectively). The difference in haemoglobin concentration was not significant (P=0.063), but according to the Cohen effect size, there was a medium to large effect (effect size 0.716).

The PCV, RBC count, haemoglobin and WBC count data from the Canfield and Shea (1988) study was compared to this study using independent t-tests or Mann Whitney U test (WBC). Scatterplots were also produced to compare the data (Figure 29-32).
Figure 29: The data for the packed cell volume of the bobtails in this study (n=40) and the Canfield and Shea (1988) study (n=6). The horizontal lines represent the reference intervals (with 90% confidence intervals) that were calculated for this study.

Figure 30: The data for the haemoglobin concentration of the bobtails in this study (n=32) and the Canfield and Shea study (n=6). The horizontal lines represent the reference intervals (with 90% confidence intervals) that were calculated for this study.
There were no significant differences in PCV (P=0.982) or haemoglobin concentration (P=0.326) in this study and the Canfield and Shea (1988) study.

Comparison of red blood cell counts yields a different result (Figure 31).

Figure 31: The data for the red blood cell counts of the bobtails in this study (n=38) and the Canfield and Shea study (n=6). The horizontal lines represent the reference intervals (with 90% confidence intervals) that were calculated for this study.

There was a significant difference between the RBC counts in this study and the Canfield and Shea study (1988) (P=0.029). There were two bobtails in the Canfield and Shea study which were 0.2-0.3 x 10^{12}/L above the reference intervals constructed in this study. The Canfield and Shea bobtails were an average of 0.32 x 10^{12}/L higher (mean difference).

There was also a significant difference between the white blood cell counts of this study and Canfield and Shea (1988) (Figure 32).
Figure 32: The data for the white blood cell counts of the bobtails in this study (n=39) and the Canfield and Shea study (n=6). The horizontal lines represent the reference intervals (with 90% confidence intervals) that were calculated for this study.

There was a significant difference between white blood cell counts (P<0.005). The WBC count of one bobtail in the Canfield and Shea study was $1 \times 10^9$/L less than the calculated lower reference interval. The median WBC count was $3.01 \times 10^9$/L lower in the Canfield and Shea study.

Finally, the correlation was calculated between total plasma protein as measured by the biuret method, and total plasma protein as measured by refractometry (Figure 33).
There were two bobtails with total protein biuret 47g/L and refractometry 50g/L. Although there was only a small sample size (n=8), the correlation was very strong (r=0.986).

Statistical analysis of data yielded several interesting results. There were multiple correlations between analytes, the majority of which were low. Blood volume was not correlated with PCV, RBC, haemoglobin or WBC. Outdoor bobtails had a lower PCV and basophil percentage and a higher heterophil percentage and degree of polychromasia than indoor bobtails. There was a statistically significant decrease in PCV associated with the presence of haemogregarines. The red blood cell counts were lower and white blood cell counts were higher in this study than that of Canfield and Shea (1988). There was a strong positive correlation between plasma total protein measured by refractometry, and total protein measured in plasma by the biuret method.

**1.2.4 Blood smear examination**

Blood smears were examined from 46 healthy bobtails. On Wright’s-Giemsa stain, the background was pale to moderately blue and proteinaceous. In some of the smears where
there was sufficient blood collected to only produce smears, the background was more dense, and surrounding the cells, the background was purple to pink. Nine morphologically distinct cell types were identified on each smear. Occasional bare nuclei were also seen on the smears but were not included in any counts.

**Erythrocytes**

The mature erythrocytes were large, ovoid, nucleated cells, which stained pink to orange on Wright’s-Giemsa stain (Figure 34). The cytoplasm stained faintly brown with a benzidine peroxidase stain (Figure 34). The erythrocytes were approximately 15µm long and 8µm wide (n=5). They had a small, central, ovoid nucleus which stained dark blue and measured 6µm long and 4µm wide (n=5). The chromatin was dense and the nuclear membrane was often variably irregular and undulating. Occasional more elongated or tapered poikilocytes were seen but no bobtails had an overt poikilocytosis (Figure 35). Within the cytoplasm of some erythrocytes, there were occasional punctate, clear, refractile, round vacuoles which were approximately 0.8µm in diameter (n=7). These vacuoles stained red (positive) with the PAS reaction (Figure 34).

![Figure 34: Mature erythrocytes stained with (left to right) Wright’s-Giemsa, benzidine peroxidase, and PAS (arrows indicate positive staining inclusions). (1000x magnification)](image_url)
Figure 35: A teardrop-shaped poikilocyte. (600x magnification, Wright’s-Giemsa)

On 13 smears (28%), within the cytoplasm of some erythrocytes, there were pale blue, ovoid inclusions with a thin capsule, a central area of pink to orange (very similar in colour to the erythrocyte cytoplasm), and a single darker blue nucleus at one pole (intraerythrocytic parasites) of approximately 7µm long by 3µm wide (n=8), (Figure 36). On four smears (9%), there were also inclusions in the erythrocyte cytoplasm with a more pleomorphic ovoid to curved shape, possessing a typically eccentric but occasionally central, dark blue nucleus of 7-17 coarse clumps (n=7), a pale blue centre, and 1-7 punctate clear vacuoles of 0.3-1.2µm in diameter (n=8) (intraerythrocytic parasites) (Figure 36). These second type of inclusions frequently caused the nucleus of the erythrocyte to be displaced eccentrically. Most erythrocytes with inclusions contained only one, but very rarely, two were seen in the same erythrocyte. On nine affected smears, the proportions of affected erythrocytes varied from 5 to 205 per 1000 erythrocytes (0.5-20.5%). On four smears, <1 per 1000 erythrocytes (<0.1%) were affected.

Figure 36: Intraerythrocytic parasites (left to right)- single infections of the ovoid morphology (2000x magnification), dual infection of the ovoid morphology (1000x magnification), and two single infections of the more pleomorphic morphology (both 2000x magnification). (Wright’s-Giemsa)
On all smears except one, there were variable numbers of cells consistent with immature erythrocytes or polychromatophils. The stage of polychromatophil was not further distinguished. Numbers varied between 0.3 to 70 polychromatophils per 1000 erythrocytes (0-7%). Polychromatophils were round cells, approximately 12-18µm in diameter (n=6), with a variably-dark blue hue to the cytoplasm. The nucleus was round, dark blue, and varied from 5-10µm in diameter (n=5). The chromatin pattern was finely clumped and open, sometimes giving a “checkerboard” appearance (Figure 37). Cytoplasmic inclusions which were seen in the mature erythrocytes, as described above, were not noted in polychromatophils. On new methylene blue stains, fine blue granules (RNA) were seen encircling the nucleus in these cells (Figure 37). On the smears from five lizards, rare mitotic figures were seen in polychromatophils (Figure 37).

![Figure 37: Polychromatophilic erythrocytes (left to right)- a polychromatophil (Wright’s-Giemsa), a mitotic figure, and reticulocytes (new methylene blue; arrows indicate the positive-staining reticulin). (1000x magnification)](image)

**Leukocytes**

The two methods of white blood cell count smear estimates were compared to one another, and to the manual WBC count (haemacytometer) using pairwise comparisons with Bonferroni post-hoc adjustment. There was no significant difference between the two smear estimate methods (P=1.000). There were significant differences between the manual count and both smear estimate methods (P=0.002 and P=0.008 for method 1 and 2 respectively). Smear estimate method 1 gave a 1-5 x 10⁹/L higher white cell count that the manual count. Smear estimate method 2 yielded a 0.7-6.1 x 10⁹/L higher white cell count.
Six different morphological types of leukocytes were readily identifiable on the smears. These leukocytes were classified as heterophils, basophils, eosinophils, azurophils, monocytes and lymphocytes.

Granulocytes

Heterophils

On the vast majority of the smears, the heterophil was the leukocyte most frequently seen. They constituted 34 to 92% of the differential count (90% confidence intervals; n=46). They were large ovoid cells of approximately 13µm in diameter (n=7). They had an eccentric, lobulated nucleus with typically 2 lobes, or occasionally 3-4 lobes (Figure 38). Occasional heterophils had an ovoid nucleus. The nucleus was approximately 12µm long and 3µm wide and stained moderately dark blue, with occasional pale blue lobes and dense chromatin (n=7). Nuclear lobes could appear disparate- seen across the cytoplasm away from the main body of the nucleus. Toxic changes were not observed. The cytoplasm stained homogenously pale pink with Wright’s-Giemsa and Leishman’s, and darker pink with the rapid stain (Figure 39). On Wright’s-Giemsa, the cytoplasm contained approximately 20-90 bright pink, round to ovoid granules which were 0.3-0.8µm long (n=8). These individual granules were not always visible on the rapid stain or Leishman’s stain. The granules stained pale brown with benzidine peroxidase stain, pink/red with PAS, very faintly blue with alcian blue, but did not stain with toluidine blue, oil red O, or new methylene blue (Figure 39).

Figure 38: Heterophils. (Left and centre 1000x magnification, right 2000x magnification; Wright’s-Giemsa)
Basophils

Basophils approximated 0-20% of the differential count (90% confidence intervals; n=45). They were ovoid cells with some variability in size but of approximately 11µm in diameter (range 7-17µm) (n=8). The nucleus was eccentric, ovoid and pale blue, of approximately 6 x 5µm in diameter (n=6) with very fine chromatin. The cytoplasm and often much of the nucleus was obscured by hundreds of round, plump, dark purple granules. The granules were approximately 0.5µm in diameter (n=8). In some cells where the granules were less numerous, individual granules appeared purple-pink (Figure 40). The granules stained purple (metachromatic) with toluidine blue, blue with new methylene blue, and pink/red with the PAS reaction. Basophil granules did not stain with alcian blue or peroxidase (Figure 41).

Figure 40: Basophils. (Left and centre 1000x magnification, right 2000x magnification; Wright’s-Giemsa)

Figure 41: Basophils stained with (left to right) rapid stain, PAS and toluidine blue. (1000x magnification)
Eosinophils

On all smears were a population of nucleated cells which constituted 0-22% of the differential (90% confidence intervals; n=46). They were the largest cells, of approximately 17µm diameter (n=7), and round to ovoid. The cytoplasm was completely filled with numerous, small, clear to pale grey vacuoles. In some cells, low numbers (<5) of very fine (<0.1µm) pale grey granules could be seen between the vacuoles. They had an eccentric, ovoid to cleaved nucleus, which stained dark blue, and a finely clumped and slightly open chromatin pattern (Figure 42). The cytoplasm stained diffusely pale blue with new methylene blue, including the vacuoles, but otherwise remained clear and vacuolated on rapid stain, Leishman’s, peroxidase, toluidine blue, alcian blue, PAS and oil red O stains (Figure 43).

Experts were consulted as to the identity of these cells\textsuperscript{1,2,3}. The unanimous conclusion was that the vacuolated cells were most likely eosinophils.

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2 Professor A. Rick Alleman DACVP (Clinical), College of Veterinary Medicine, University of Florida, USA.
3 Dr. Nicole I. Stacy DACVP (Clinical), College of Veterinary Medicine, University of Florida, USA.

Figure 42: Eosinophils with occasional very small granules (arrows). (Left and centre 1000x magnification, right 2000x magnification; Wright’s-Giemsa).

Figure 43: Eosinophils with rapid stain (left) and new methylene blue (right; note the intraerythrocytic haemogregarine (arrow)). (1000x magnification)
On the smears from two bobtails, very rare, round leukocytes were seen which possessed a low nuclear to cytoplasmic ratio, an eccentric ovoid nucleus, finely clumped chromatin, and a pale cytoplasm which was filled with large, round magenta granules (presumed granulated eosinophils) (Figure 44). Too few cells were present for cytochemical examination.

Figure 44: Nucleated cells with magenta granules (presumed granulated eosinophils) and a vacuolated eosinophil (right). (1000x magnification, Wright’s-Giemsa)

**Mononuclear cells**

Monocytes were seen as two morphological entities: the azurophil and the non-azurophilic monocyte. They were counted together to yield the (total) monocyte count which comprised 1-27% of the differential count (90% confidence intervals; n=45). The two morphologies will be described separately.

**Azurophils**

The most frequently seen monocyte was the azurophil. Azurophils were round cells which ranged from 9 to 13µm in diameter (n=8). They had an ovoid to bilobed, often eccentric nucleus with dark blue, fine chromatin. The cytoplasm was pale blue-grey and contained a fine dusting of variable numbers of bright pink to purple granules of <0.1µm in diameter (Figure 45). These fine granules did not obscure the nucleus. The granules stained very faintly with PAS (Figure 46). The cytoplasm stained diffusely pale purple (metachromatic) with toluidine blue (Figure 46). Neither the cytoplasm nor the granules stained with peroxidase, alcian blue or oil red O stains.
A single azurophil was seen with a phagocytosed erythrocyte in the cytoplasm on a smear prepared by Vetpath Laboratory Services (Figure 46). This smear was made approximately 24 hours following blood collection.

Figure 45: Azurophils. (Left 1000x magnification, right 2000x magnification; Wright’s-Giemsa)

Figure 46: Azurophils with (left to right) a phagocytosed erythrocyte (Wright’s-Giemsa), rapid stain, toluidine blue (with an eosinophil below) and PAS. (1000x magnification)

**Monocytes**

Monocytes were seen occasionally on the smears. They were round cells of approximately 11µm in diameter (n=5). The nucleus was dark blue, bilobed to irregular, 10µm long and 5µm wide (n=5), with fine chromatin. The cytoplasm was pale blue-grey and homogenous; vacuoles were not seen (Figure 47). The cytoplasm did not stain with peroxidase, toluidine blue, alcian blue, oil red O or PAS.
Lymphocytes

Lymphocytes comprised 2-15% of the leukocyte differential count (90% confidence intervals; n=41). They were round cells of variable size from 7µm to 13µm (n=12). Unlike other leukocytes where the nuclear to cytoplasmic (N:C) ratio was low to moderate, but consistent in each cell type, the lymphocyte N:C ratio varied from subjectively low to high. The nucleus was round, dark blue with a fine chromatin pattern, varying from 5 to 10µm in diameter (n=12) (Figure 48). The cytoplasm was typically pale blue and homogenous, although occasional lymphocytes had a darker blue cytoplasm (reactive) (Figure 49). A few, 1-2µm clear punctate vacuoles were seen in the cytoplasm of some lymphocytes. Larger lymphocytes occasionally had a single prominent, large nucleolus (lymphoblasts). A morphological variant occasionally seen was a lymphocyte with a low N:C ratio and an area of perinuclear clearing in the cytoplasm (plasmacytoid) (Figure 49). Other, rare lymphocytes contained approximately ten fine, bright pink cytoplasmic granules within the cytoplasm (presumed lysosomal granules) (Figure 49). On one smear, a single lymphocyte with multiple, 1µm diameter, discrete, round pale pink bodies in the cytoplasm was seen (presumed Russell bodies within a Mott cell) (Figure 49). Lymphocytes did not stain with peroxidase, toluidine blue, alcian blue, oil red O or PAS.
Thrombocytes

Thrombocytes were seen singly and in clumps on the smears (Figure 50). Due to the clumping, thrombocyte counts were not attempted. The thrombocyte nucleus was round, dark blue, with fine, very dense chromatin. The cytoplasm was typically very pale blue, often with poorly defined or wispy margins, which gave the cell a round, irregular or elongated form. Vacuoles were sometimes seen within the cytoplasm (Figure 50). The nuclear to cytoplasmic ratio was high, with the cytoplasm approximately 7µm in diameter (n=7) and the nucleus 6µm in diameter (n=8). Thrombocytes were frequently present just as bare nuclei. On smears stained with the PAS reaction, the cytoplasm of the thrombocytes contained few (3-10) punctate, red granules (Figure 51). Thrombocytes did not stain with peroxidase, toluidine blue, alcian blue, or oil red O.
1.2.5 Plasma biochemistry

The plasma was clear to very pale yellow for all lizards. Plasma concentrations of total protein, albumin, inorganic phosphate, total calcium, glucose and uric acid were measured in eight bobtails (Table 11).
There were insufficient sample numbers to calculate reference intervals for the plasma biochemistry. There was a wide range of measured uric acid concentrations. The concentrations of other analytes were less variable. Bobtail 48 had a lower total protein than the other bobtails, and this was due to a lower albumin concentration. The blood glucose concentration of bobtail 49 was higher than the other lizards.

1.2.6 Transmission electron microscopy

Stained ultrathin sections of the cell pellets from four healthy bobtails were examined by transmission electron microscopy. The majority of the cells present on the grids were mature erythrocytes with leukocytes appearing in similar quantities to those on the blood film. Thrombocytes were also present and were seen singly and in small clumps.

The erythrocytes were ovoid to fusiform cells with a central, large, ovoid nucleus and abundant heterochromatin. The cytoplasm was dark grey and homogenous, and occasional organelles such as mitochondria and endoplasmic reticulum were seen (Figure 52).
In rare cells, there was a single large cytoplasmic inclusion consistent with an apicomplexan, haemogregarine gamont enclosed within a parasitophorous vacuole (Figure 53). The gamonts were ovoid, of approximately 3µm x 2µm, and were surrounded by a thin, double-layered plasmalemma. A small nucleus with a prominent nucleolus was present and the cytoplasm was heterogeneous and finely granular. Multiple, variably-sized, round to ovoid granules (micronemes) were seen within the cytoplasm, particularly around the periphery. These micronemes were variable in their electron density.

Also seen were rare large cytoplasmic inclusions similar to that described above, but with a large, distinct nucleus and multiple, uniform, small vacuoles (Figure 54).
Figure 53: Transmission electron microscope photomicrographs of haemogregarine gamonts within the erythrocyte cytoplasm. Arrows indicate micronemes (with a clear apical complex in the top right image), the arrowhead indicates the nucleus. Processing has caused some distortion of the gamonts. (Top left 3810x magnification, top right 6500x magnification, bottom left 4800x magnification, bottom right 20,000x magnification; uranyl acetate and lead citrate)
As on the blood films, the most numerous leukocytes present on the grids were heterophils. Heterophils were round cells with either a single, eccentric ovoid nucleus or a nucleus with two or three disparate lobes. There was moderately abundant heterochromatin which was typically peripheral. The cytoplasm contained many round to ovoid to elongate granules of variable sizes and electron densities (Figure 55). The cytoplasm was pale to medium grey and finely granular, with scattered organelles such as mitochondria and endoplasmic reticulum.
Lymphocytes were frequently seen. They were round cells with a high nuclear to cytoplasmic ratio and the nucleus was round to indented, central, and contained a small to moderate amount of heterochromatin. The cytoplasm was scant, medium grey and finely granular with a few, moderately electron-dense small granules, and occasional organelles (Figure 56).
Occasional monocytes were seen. They were round cells with a low to moderate nuclear to cytoplasmic ratio. The nucleus was central or eccentric, ovoid, with a low to moderate amount of heterochromatin. The cytoplasm was medium grey and there were variable numbers of small, moderately electron-dense granules (azurophils), and large numbers of organelles (mitochondria, endoplasmic reticulum, Golgi apparatus) (Figure 57).

Figure 56: A transmission electron photomicrograph of a lymphocyte with a single small granule in the cytoplasm (arrow). (8400x magnification; uranyl acetate and lead citrate)

Figure 57: Transmission electron photomicrographs of monocytes. The monocyte on the left only has few granules (arrow) and the monocyte on the right has a moderate number of granules (presumed azurophil). (8400x magnification; uranyl acetate and lead citrate)
Eosinophils were very occasionally seen on the grids. They were round cells with a small, eccentric, ovoid nucleus, and a moderate amount of peripheral heterochromatin. The pale to medium grey cytoplasm contained many large, clear vacuoles, some of which contained a single rod-shaped, crystalline inclusion (Figure 58). Occasional large granules were seen which were of low to moderate electron-density. There were also scattered small, electron-dense granules present between vacuoles and few distinct organelles such as mitochondria.

Figure 58: A transmission electron photomicrograph of an eosinophil with crystalline inclusions in vacuoles (white thick arrow). Higher magnification (right) of a vacuole (asterisk), an intact large granule (arrow) and a small granule (arrow head). (Left, 8400x magnification, right, 74,000x magnification; uranyl acetate and lead citrate)

Despite examination of multiple sections from multiple samples, basophils could not be identified.

Single thrombocytes and small thrombocyte clumps were scattered across the grids. They were round to ovoid cells which often had projections from the cytoplasm. The nuclear to cytoplasmic ratio was moderate to high, possessing a central, irregular, folded nucleus with a moderate amount of peripheral heterochromatin. The cytoplasm was medium grey and contained prominent organelles (mitochondria, endoplasmic reticulum, Golgi apparatus). Encircling the cytoplasm, there were elongated structures of pale grey to white, lined by a thin membrane (canalicular structures) (Figure 59). Small and moderately electron-dense
granules were scattered in the cytoplasm and occasional small clear vacuoles were also seen.

Figure 59: Transmission electron photomicrographs of thrombocytes.
Top left- A clump of nine thrombocytes. Note the cytoplasmic projections, and few vacuoles (2850x magnification).
Top right- A single thrombocyte with prominent organelles and a few visible canalicular structures (arrow) (8400x magnification).
Bottom- A single thrombocyte with prominent canalicular structures (arrows) (8400x magnification). (Uranyl acetate and lead citrate)
1.3 Discussion

1.3.1 Complete blood count

Assessment of the utility of the reference intervals

The majority of the reference intervals calculated from the data were very wide, reflecting the variation in results. This meant that the reference intervals with 90% confidence intervals, which highlights uncertainty in the data, were wider still. It is likely that there are multiple reasons for the large variability in results including small sample size, captivity, differences in ambient temperature, sex, and possibly reproductive status and diet (Anderson et al., 1997; Campbell, 2006; Cartledge et al., 2005; Harr et al., 2001; Jacobson and Origgi, 2002; Montali, 1988; Salakij et al., 2002).

One important consideration is the sample size. It is recommended that meaningful haematological reference intervals should be derived from 120 subjects, or 40 at the least (Friedrichs et al., 2011). The population size of 52 bobtails was selected as this approximated the number of healthy, adult, captive Tiliqua rugosa available for sampling in Perth, Western Australia. A larger sample size would have yielded reference intervals that more accurately reflects the population; however the study was limited by the number of bobtails available. Captive lizards rather than wild caught lizards were selected for this study for several reasons. The captive lizard population is readily available for sampling. Sampling wild bobtails requires trapping. Trapping induces significant stress, which may alter haematological results (Campbell and Ellis, 2007; Moore and Jessop, 2003; Salakij et al., 2002). The sampling and venepuncture procedure itself does cause acute stress also, but this would be less of an issue in the captive lizards which are used to handling. In captive lizards, the owners/carers monitor their health status; hence sampling captive lizards improves the chance that the reference population is truly healthy. However, the use of captive lizards in this study may limit the use of the reference intervals to only captive bobtails. The intervals may not be applicable for wild caught lizards, with factors such as stress (acute in wild caught vs chronic in captive lizards), ambient temperature and diet.
likely to cause differences in the haematology (Cooper-Bailey et al., 2011; Salakij et al., 2002).

Another reason for the wide reference intervals may be differences in preanalytical factors. It is well-established that preanalytical factors have an influence on haematological results in reptiles (Campbell and Ellis, 2007). Where possible in this study, preanalytical factors were controlled: season, venepuncture site, anticoagulant, and smear staining were uniform for all bobtails sampled. The housing of bobtails did vary in this study, with either heated vivariums, semi-heated enclosures, or open, outdoor enclosures. Bobtails housed outdoors had a slightly lower PCV (mean difference of 0.06L/L) and a slightly higher degree of polychromasia (mean difference of 1.1%). The reason for this difference is not clear. If the effect was due to a difference in ambient temperature, then the PCV of the outdoor bobtails would be expected to be higher due to haemoconcentration. At the lower end of the PCV reference interval, a difference of 0.06L/L, which approximates one standard deviation (0.07L/L), may be clinically significant. While PCV and polychromasia had a weak negative correlation (r=-0.329), a difference of 1.1% in the polychromasia is unlikely to be clinically significant.

The heterophils were higher in the outdoor bobtails by 8.4%, and basophils were lower by 3.5% (mean differences). Given the very wide reference interval of 27-88% for heterophils, this difference is unlikely to be clinically significant. The basophil reference interval was also wide and non-parametric, 0-20%, and likewise, clinically, this is unlikely to be significant.

The bobtails did also vary with their time in captivity, but all bobtails except one had been in captivity for months to years. Captivity causes stress. Bobtail Healthy 32 had been in captivity for a few weeks. When the reference intervals and associated data were recalculated following exclusion of this bobtail, the means varied very little. The lower reference interval for haemoglobin decreased by 5.5g/L as the haemoglobin of this bobtail was one of the higher values (103g/L; upper reference interval 154g/L). The upper reference interval for MCH and MCHC were also decreased. Following exclusion, the upper reference interval for eosinophils decreased by 2.8%. For all other reference intervals, the effect was negligible. Not a lot can be derived from this as it was only one animal, and further studies
on recently captured bobtails would be required to evaluate further. There are differences in the physiological response to acute and chronic stress as they are mediated by separate hormones (chiefly adrenaline and corticosterone in reptiles) (Cooper-Bailey et al., 2011; Salakij et al., 2002). It is not known how length of time in captivity correlates with stress, and the types of hormonal responses elicited in bobtails.

With regards to intrinsic or biological preanalytical factors, only adult bobtails were included (with one possible exception), and only those that were clinically healthy, as determined by examination and discussion with owners/carers, were sampled. There were uncontrolled intrinsic factors in this study: namely sex, diet and possibly reproductive status. Bobtails exhibit a low degree of sexual dimorphism and manual eversion of hemipenes is difficult and stressful, thus the sex of the lizards was not known. Visibly gravid bobtails or those known or suspected to be pregnant by the carers were not sampled, but the sampling season does coincide with the breeding season, so it is possible that some bobtails included in this study were pregnant. Sex and reproductive status can influence the haematological results in many reptile species (Anderson et al., 1997; Chansue et al., 2011; Harr et al., 2001; Strik et al., 2007). Stratification of reference intervals for male and female bobtails would likely narrow the reference intervals (given adequate sample sizes), however it was considered that manual eversion of hemipenes would not be attempted on every bobtail presented for examination at veterinary clinics and wildlife centres, and thus non sex-specific intervals would be more clinically useful.

The diet varied between the locations from which the bobtails were sourced. Further information was not obtained. All bobtails would have been fed a high quality diet as they were cared for by experienced reptile owners. Most lizards were group-housed, so it is possible that competition for food may have resulted in some lizards receiving less of some food types; however, experienced reptile owners are typically vigilant at monitoring this. A suboptimal diet can be a source of stress (see above) or cause nutritional deficiencies such as iron deficiency which may affect haematology (Campbell and Ellis, 2007).

Despite the width of the reference intervals, they can be used as guidelines for the haematological assessment of captive bobtails. It has been suggested that because of the

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inherent variability with reptile haematology, only two-fold or above increases or decreases from the reference intervals are considered significant (Campbell, 2006). Whilst this may be debatable, the use of reference intervals with 90% confidence intervals does take the imprecision and variability of the data into account (Braun et al., 2013). Although caution in interpretation is still warranted, it is this author’s suggestion that using the same methodology, these reference intervals with 90% confidence intervals can be applied for the haematological assessment of captive, adult *Tiliqua rugosa* in summer.

### Evaluation of the accuracy of the methodology

In order for the complete blood count reference intervals to be valid, it is important to ensure that the methods used in this study were appropriate for the analyses performed and also were applicable for this species.

To the author’s knowledge, use of the PICO50 syringes for blood collection and haematological analysis of reptiles has not been previously reported. The PICO50 syringes contain a disc of 80IU, dry, electrolyte-balanced lithium sodium heparin. These syringes are designed for use in humans but are also used in animals for blood gas and electrolyte analysis. The concentration of heparin in the syringes is optimised for preventing coagulation of 2mls of blood. When the syringe is partially filled to 0.5ml, there is a very mild, yet significant decrease in plasma sodium concentration of approximately 2mmol/L according to one study (Yip et al., 2006). Acute, pathological hyponatraemia of dogs and cats causes cell swelling, increasing the PCV and measured MCV (Boisvert et al., 1999). Thus it was considered possible the same occurred in this study. However, the statistics do not concur, as the PCV is not significantly increased in lizards where 1ml or more of blood was collected when compared to less than 1ml (P=0.760), nor at above or below 0.5mls (P=0.935). The MCV was a calculated value in this study so a comparison was not made. There was also no effect of low blood volume on the haemoglobin concentration, red blood cell or white blood cell counts. The PICO50 lithium heparin syringes appear to be very useful for reptile haematological and biochemical analyses. Further studies comparing to EDTA
blood for haematology, and serum for biochemistry would further evaluate the utility of this anticoagulant for bobtails.

The correlation analyses which were performed for all data reinforce the validity of the methods used in this study. As would be expected, PCV was positively correlated with haemoglobin concentration ($r= 0.880$), but the red blood cell count ($r= 0.446$) was less well correlated. The inherent error in the manual red blood cell count (see below) is a likely reason why the red blood cell count and PCV were not more correlated. Also the limited sample size would have influenced results. The PCV was also negatively correlated with polychromasia ($r=-0.329$). This correlation was low. The low correlation was likely affected by the low degree of polychromasia seen, given they were an apparently healthy population. An influence by the haemogregarine parasites was also present (see below for further discussion). Thus it was considered that all of the methods used were suitable for the bobtail blood samples.

Manual cell counting methods are generally used in reptiles as their nucleated red blood cells and thrombocytes generally preclude automated analysis, and the automated instruments validated for use in reptiles are not available at every clinical pathology laboratory. In the repeatability study performed, the CV of the red blood cell count was approximately 10%. Coefficients of variation for manual methods have been reported as between 13% and 40%. There was a moderate correlation ($r= 0.500$) between the counts in either side of the haemacytometer chamber on any given aliquot of the same sample. All data fell within two standard deviations. Although this study was limited by its small sample size, this method appeared to be repeatable, with a CV of 10% considered reasonable (Vap et al., 2012).

The white blood cell counts were much more variable, with a CV of 20% and no correlation between counts on either side of the chamber ($r= -0.147$). Two data points fell outside greater than two standard deviations on the Bland-Altman plot. This low repeatability is likely partially responsible for the very wide WBC reference interval. Given that this method
was the same as that used for the red blood cell counts, it may be that the difference is attributable to the characteristics of the white blood cells themselves or the identification thereof, rather than the method. It is also possible that thrombocytes were erroneously included in the manual count. Size was the main factor used to differentiate thrombocytes from leukocytes. Thrombocyte clumps would be identified in the chamber and the size of individual thrombocytes re-examined where there was concern about the differentiation. However, some lymphocytes are only slightly bigger than thrombocytes (7µm and 6µm in diameter on blood smears). Thus this is one likely source of error.

White blood cell count estimates on the blood smear were also investigated as a method of obtaining a WBC count. Both methods performed gave statistically similar results (P=1.000). The WBC smear estimates were up to 6 x 10⁹/L higher than the haemacytometer count. As there was so much variability with the haemacytometer white cell counts (CV 20%), it is difficult to truly validate the smear count against it, but it is the only established method available. A cautious approach to interpretation is recommended. At lower white cell counts, the smear estimate may not be accurate. At higher cell counts, given the width of the WBC reference interval, a difference of 6 x 10⁹/L is less clinically significant, and thus a white cell count smear estimate is more likely to be useful.

Due to the limited availability of automated haematology analysers validated for use in reptiles, much of reptile haematology is analysed using manual methods. Although there is greater inherent variability in manual methods compared to automated methods, these manual methods are well established for use in non-mammalian vertebrates, and are also appropriate for the haematology of bobtail lizards.

**Specifics of the complete blood count**

These results are only applicable to the summer season. As they are ectothermic animals, seasonality has an effect on the haematology of reptiles and a separate set of reference intervals is likely required for winter (Campbell, 2006; El Ridi et al., 1988; Montali, 1988).
Examination of the haematology reference intervals, the statistical analyses performed there-in, and comparison to previously published ranges in reptiles yielded several interesting findings.

The lower limit of the PCV reference interval (0.10L/L) was much lower than the normal PCV typically reported for healthy reptiles, which is between 0.20 and 0.40L/L. Similarly, the red blood cell count in this study of 0.3-1.0 x 10^{12}/L was also lower than the reported typical range of 1 to 1.5 x 10^{12}/L (Campbell, 2006). It is not clear whether this is just a physiological variation, or whether it is pathological. Comparison to wild bobtails would be particularly useful to further interpret this.

There was a very wide variation in haemoglobin concentration of the healthy bobtails of 26-126g/L. This produced an accordingly wide reference interval. The normal haemoglobin concentration of reptiles is reported as between 60 and 100g/L (Campbell, 2006). For bobtails, the values at the lower range reflected the lower PCV and RBC counts. The higher values may simply reflect physiological variation, however as the haemoglobin was typically measured 24-36 hours after collection, some mild sample haemolysis cannot be excluded.

The MCV, MCH and MCHC are calculated values and their variability reflected that in the PCV, RBC count and haemoglobin.

The white blood cell count reference interval was also very wide, reflecting a range from 2.9 to 22.7 x 10^{9}/L. The normal white blood cell count of reptiles is 5-15 x 10^{9}/L, with stress leukograms expected as up to 20 x 10^{9}/L and greater than 30 x 10^{9}/L generally indicating bacterial infection or leukaemia (Campbell, 2006; Tocidlowski et al., 2001). It is likely that stress elevated the white cell counts of some bobtails. The reason for the lower values is not known, and low values were also seen in the Canfield and Shea (1988) study so this may be physiological (although time of year that samples were collected in that study was not
specified). The white blood cell count smear estimates also had low lower limits of 3.4 and $2.0 \times 10^9/L$ for methods 1 and 2 respectively. Thus the lower WBC counts were not due to an error in manual haemacytometer counting. Given that the reference intervals incorporate uncertainty with the 90% confidence intervals, especially around the upper reference limit of this analyte, if the white blood cell count of a captive bobtail was above the upper limit, it would suggest inflammation/infection (or possibly leukaemia).

The polychromatophil count was used as the index of red cell regeneration in this study, as this is readily apparent on smears. The degree of polychromasia was slightly higher in the bobtails compared to the count of 1% or less indicated as normal for most lizards (Campbell, 2004). A total of 26 out of the 46 bobtails possessed a polychromatophil count of more than 1%. Utilising the Campbell (2006) classification scheme of polychromasia, 31 counts were classified as nil (<2%), and the remaining 15 were classified as slight (2-10%). Thus a slight polychromasia was considered unlikely to be clinically significant.

The largest polychromatophil count was 15%, and this lizard had a PCV of 0.22L/L, near to the mean of 0.26L/L. This polychromatophil count was considered an outlier and removed from the data set. There were haemogregarine parasites on the smear from this bobtail, but the count was only 10 parasites per 10,000 red blood cells (some bobtails had up to 20 times more) and the leukocyte counts were also close to the mean values. This bobtail was also not undergoing ecdysis. The reason for the increased red cell turnover in this bobtail is not clear but it appeared to be compensated.

The reference interval (with 90% confidence intervals) for total plasma protein was the same as the range of plasma total protein, and also serum total protein, previously reported for reptiles (Proverbio et al., 2012; Stein, 1996). It has been stated that the total plasma protein by refractometry may not be reliable in reptiles, and that as in mammals, the biuret method of total protein measurement is preferred (Strik et al., 2007). This is due to the potential influence of other solids including lipids, triglycerides (lipaemia), haemoglobin (haemolysis), glucose and urea (in mammals), and inflammatory proteins (Cray et al., 2008; George, 2001). Total protein by the biuret method was measured on the plasma of eight
healthy bobtails. This sample size was very small but there was a strong correlation (r=0.986) between the two methods. The total protein by the biuret method was consistently 1-5g/L (median 3.5g/L) lower than the total plasma protein by refractometry. This disparity may be due to the presence of other solids such as fibrinogen in the plasma (Proverbio et al., 2012). This suggests that the total plasma protein by refractometry is a reasonable method of determining the total protein concentration in this species.

The 200-cell leukocyte differential counts were performed at the end of all blood collections, over four days. Similarly, polychromatophil counts and haemogregarine parasite counts were also performed on all smears over several days. This was to minimise chronological bias in the counting.

As the total white cell count reference interval was wide, it was deemed that the leukocyte differential counts were more clinically useful when expressed as a percentage, rather than as absolute counts, and as such, intervals were produced for the percentages. This needs to be considered when using the reference intervals to interpret results.

Azurophils and monocytes were counted separately in the differential counts, but combined for the final counts as azurophils are considered to be a monocyte subtype and further differentiation is not considered clinically useful (Campbell, 2006).

Some reference intervals produced in this study deviated from the normal reptile ranges quoted by Campbell (2006). Further studies with a larger sample size would further investigate whether these changes are normal and representative of this species.
1.3.2 Blood smear examination

**Romanowsky and Romanowsky-type stains**

Examination of Romanowsky-stained blood smears from healthy captive *Tiliqua rugosa* identified several interesting features.

Cell identification was uncomplicated for most of the cells on the smears but there was initial difficulty identifying the large cells with the vacuolated cytoplasm (Figure 42). These cells were identified as eosinophils. Other initial considerations for these cells were degranulated heterophils or vacuolated macrophages. Lipid-laden macrophages have been reported in blood smears from reptiles previously, however the vacuolated cells in this study were oil red O negative, making this unlikely (Stacy et al., 2011). With respect to degranulated heterophils, the nucleus was most often ovoid in these vacuolated cells, and sometimes bilobed, whereas the majority of heterophils had a bilobed or multilobulated nucleus, thus degranulated heterophils were not favoured. The eosinophils of the green turtle have a vacuolated appearance on blood films due to the presence of grey cytoplasmic granules (pseudo-vacuoles) (Work et al., 1998). In loggerhead turtles, the eosinophils are highly vacuolated (true vacuoles), and morphologically similar to those seen here in the bobtails, but most cells still contain at least a few distinct pink granules (Casal and Orós, 2007).

No other cells morphologically consistent with the eosinophils of other reptile species were identified consistently on the smears. Nucleated cells with large magenta granules were seen very rarely on two smears, and given the similarity in size, shape, and nuclear morphology, they likely represent the non-vacuolated, granulated form of eosinophil. Thus it was concluded that the vacuolated cells were most likely eosinophils. These eosinophils may be similar to those found in the green turtle, and also many adult greyhound dogs where there appears to be a difference with granule stain uptake such that the cells just appear vacuolated (pseudo-vacuoles) (Iazbik and Couto, 2005; Rizzi et al., 2010). The cytoplasm of the eosinophils stained homogenously with new methylene blue rather than remaining clear, which may further support this theory. In addition, identical morphology was seen in the blood smears prepared without anticoagulant by Smallridge (pers. obs.),
ruling this out is as consequence of lithium heparin anticoagulant use. Transmission electron microscopy was required to further evaluate the content of these vacuoles (see 1.3.5).

The other main area of difficulty with smear evaluation was that individual round thrombocytes were occasionally difficult to differentiate from small lymphocytes (Figure 50). The features used most often to differentiate them were the density of the chromatin: very dense for thrombocytes and less so for lymphocytes, the colour of the cytoplasm: very pale grey/blue for thrombocytes and darker for lymphocytes, and the cell margins: typically discrete for lymphocytes and less well-defined for thrombocytes.

The clear, refractile vacuoles seen in the cytoplasm of erythrocytes were consistent with artefacts most often ascribed to either slow drying or smear preparation (Reagan et al., 2008). The smears were prepared outdoors during summer with variable but typically low humidity, thus slow drying is considered less likely and smear preparation is the more likely reason.

The large cytoplasmic inclusions seen in erythrocytes from 13 lizards were consistent with haemogregarine parasites. Parasites were only seen within mature erythrocytes and not within any other cells on the smears. Two morphological entities were seen. The most common form was an ovoid gamont with a blue nucleus at one pole, a homogenous pink to orange area in the centre, and a surrounding pale blue capsule. In four smears, the more pleomorphic merozoites were seen, possessing an ovoid to curved shape, a nucleus with coarse chromatin clumps, and punctate clear vacuoles. Haemogregarine parasites cannot be distinguished by their gamont morphology alone, but *Hemolivia mariae* has been reported in this species previously. Both encapsulated and unencapsulated *Hemolivia mariae*, which appear identical to the morphologies seen in these bobtails, have been previously been reported and studied (Smallridge, 1998). Smallridge (1998) concluded that the unencapsulated form most likely matured to the encapsulated form. Thus it is considered likely that the parasites seen were *Hemolivia* or a closely related species.
Occasional reactive lymphocytes were seen on many smears. Rarely seen were granular lymphocytes, plasmacytoid lymphocytes and lymphoblasts. A single lymphocyte with multiple pale pink round bodies in the cytoplasm was morphologically consistent with a Mott cell (Figure 49). Reactive lymphocytes may occasionally be seen in healthy reptile blood smears, but are usually seen associated with antigenic stimulation due to inflammation or infection (Rovira, 2010; Stacy et al., 2011). Of the 31 out of 46 smears which contained some reactive lymphocytes, 12 also had haemogregarine parasites, but 19 did not. Only one lizard with haemogregarines did not have immunoreactive lymphocytes on the smear. It is thus possible that haemogregarine infection is one cause of chronic antigenic stimulation. There was no correlation between reactive lymphocytes and any other haematology variable.

There was a low positive correlation between haemogregarine parasite infection and polychromasia ($r=0.396$). There was also a significant difference between the PCV of infected and non-infected lizards, with a slightly lower mean PCV of 0.04L/L in infected lizards. There was no significant difference ($P=0.063$) but a moderate to large Cohen effect (effect size 0.716) of the presence of haemogregarines with haemoglobin concentration. These data suggest that haemogregarine infection causes erythrocyte pathology resulting in stimulation of erythropoiesis. Haemolysis is considered the most likely reason. There was also a low but negative correlation with infection and total plasma protein by refractometry ($r=-0.370$). The reason for this is not known as an increase in globulins and therefore total protein may be expected with chronic antigenic stimulation, rather than a decrease as seen here. Larger numbers of infected lizards need to be examined to further investigate these correlations.

There was variable basophilia of the heterophil nucleus with the Romanowsky and rapid stains, which is also seen in other lizard species such as the iguana (Rovira, 2010). Possibly one of the chemical components of the heterophil cytoplasm such as myeloperoxidase is preventing the acidophilic dye from properly binding to the nucleic acids within the nucleus.
One azurophil was seen with a phagocytosed erythrocyte in the cytoplasm (Figure 46). This smear was made following refrigerated storage of the blood for 24 hours. There was no evidence of erythrophagia on the smear prepared directly after blood collection from this lizard. It is likely that erythrophagia occurred in vitro during storage and not in vivo, which is further supported by the aging changes of the cells on this smear. It does indicate that azurophils are capable of phagocytosis, consistent with previous reports (Heard et al., 2004; Jaensch and Raidal, 2006).

There were staining differences in the leukocytes between the Romanowsky-type stains. The greatest difference was in the staining of heterophil granules where distinct granules could be seen with the Wright’s-Giemsa stain but only in occasional cells on the rapid stain. The cytoplasm of the heterophils stained homogenously a much darker pink on the rapid stain. This difference has been seen in other reptile species (Campbell and Ellis, 2007). The reason why the eosin in the rapid stain has a stronger binding affinity for the heterophil cytoplasmic proteins than the eosin in the Wright’s-Giemsa is not known, but may be due to differences in the pH of the solution or differences in the buffers.

On Wright’s-Giemsa-stained blood smears, healthy bobtails in this study had vacuolated eosinophils, thrombocytes and small lymphocytes which appeared similar, refractile bodies in the cytoplasm of erythrocytes, variable basophilia of the nucleus of some heterophils and occasional reactive lymphocytes. Some bobtails had circulating intraerythrocytic haemogregarine parasites, and there was a low yet positive correlation between the presence of parasites and polychromasia, suggesting that their presence was causing stimulation of erythropoiesis, possibly secondary to haemolysis. A phagocytic azurophil was seen in one smear, supporting that their function in bobtails is the same as in other reptiles. Finally, staining differences were noticed between Wright’s-Giemsa and the rapid stain, with the most prominent difference an enhanced eosinophilia of the heterophil cytoplasm on the rapid stain.
Cytochemical stains were applied to unstained, air-dried blood smears in an attempt to characterise some of the cellular constituents of the cells, enable a comparison with other reptile species, and in some instances, assist with cell identification (Raskin, 2010).

Granulocytes

Heterophils

The positive reactions with benzidine peroxidase, PAS and alcian blue characterise the contents of the heterophil cytoplasm.

Individual heterophil granules stained red/brown with benzidine peroxidase. The benzidine peroxidase reaction involves the oxidation of benzidine by exogenous hydrogen peroxide, catalysed by cellular peroxidase (myeloperoxidase). The oxidised benzidine appears initially blue, then persists as a red/brown granular colour in the area the reaction has occurred (Lynch et al., 1969). There are many types of peroxidases, but the enzyme that is present in the largest concentrations in the granulocytes of most species is myeloperoxidase; thus this stain is considered a stain to detect myeloperoxidase. Other endogenous peroxidases such as cytochrome oxidase and catalase can potentially cause cross reactions (Kiernan, 1999). Many reptile heterophils do not stain positive with benzidine peroxidase and are thus considered not to contain myeloperoxidase and therefore not capable of killing microorganisms with oxidative activity (Raskin, 2010). Thus it may be considered that the heterophils of bobtails possess myeloperoxidase and are likely capable of the oxidative burst, however this must be verified by phagocytic function testing (Harr et al., 2001). Other reptiles possessing heterophils which stain positive for peroxidase include the green iguana, rainbow lizard, black and white tegu lizard and the yacare caiman (Carvalho et al., 2006; Caxton-Martins and Nganwuchu, 1978; Harr et al., 2001; Oliveira et al., 1998).
A positive reaction of the heterophil cytoplasm with the PAS reaction suggests the presence of glycoproteins. This is consistent with the content of most reptile heterophils and the neutrophils of domestic mammalian species (Nabity and Ramaiah, 2010; Raskin, 2010). Glycogen is the most common PAS-positive carbohydrate found in the cytoplasm of blood cells and is utilised in the generation of ATP. A PAS-positive reaction is not entirely specific for glycogen as other polysaccharides, glycoproteins, glycolipids, or mucoproteins will also react and stain (Jain, 1986; Lynch et al., 1969).

Heterophil granules stained weakly with alcian blue. Alcian blue is a dye with electrostatic attraction to acidic residues of acid mucopolysaccharides. Alcian blue is typically used on blood smears to stain the sulphated mucosubstances present in the basophils of many species (Kiernan, 1999; Lynch et al., 1969; Raskin, 2010). Sulphated and carboxylated connective tissue mucins also stain positive at the standard pH of 2.5 (Kiernan, 1999; Lynch et al., 1969). The positive reaction of boxtail heterophil granules suggests the presence of mucopolysaccharides such as glycosaminoglycans within the granules. Glycosaminoglycans are present in primary granules of human and guinea pig neutrophils and rabbit heterophils, where they are thought to have a role in the binding and inactivation of stored enzymes (Hardin and Spicer, 1971; Parmley et al., 1983; Yang et al., 1999). Toluidine blue and alcian blue also stain the azurophilic granules seen in the neutrophils of animals with mucopolysaccharidoses, a group of genetic disorders of mucopolysaccharide catabolism (Haskins et al., 1983; Weiss, 2010). Interestingly, the heterophil granules of boxtails stained with alcian blue but not with toluidine blue. The type of mucosubstance present in the heterophil granules is not known.

**Eosinophils**
The only stain that was taken up by the eosinophils was new methylene blue. New methylene blue is a basic dye which stains acidic structures, mostly proteins or protein-linked substances (Lynch et al., 1969). The staining of the eosinophil cytoplasm and vacuoles was similar to the background staining so more information about the cytoplasm was not obtained. Given the narrow spectrum of cytochemical stains applied to the smears, the
contents could not be further typified. Additional stains such as Luna, acid phosphatase, chloroacetate esterase, alpha-naphthyl butyrate esterase and Sudan black could assist with characterising the cytoplasm contents.

Unfortunately, the nucleated cells with the large magenta granules - the non-vacuolated eosinophils - were too rare to be cytochemically characterised.

**Basophils**
Basophil granules stained with PAS, suggesting the presence of glycogen or other carbohydrate in the granules. The granules also stained metachromatic with toluidine blue. Toluidine blue is a basic dye which, at an acidic pH, stains acid polysaccharides such as heparin, sulphated glycoproteins (such as mucus) and proteoglycans (Kiernan, 1999). Metachromasia is a feature of the granules of basophils and mast cells of mammals and some reptiles when stained with toluidine blue. Metachromasia results from the formation of polymers which are produced by the binding of large amounts of toluidine blue by the large anionic molecules within the granules (Lynch et al., 1969). Positive staining suggests that basophil granules of bobtails contain heparin, and therefore may have a role in haemostasis, similar to mammalian basophils (Pohlman, 2010).

**Mononuclear cells**

**Azurophils**
The cytoplasm of the azurophils stained weakly with the PAS reaction, suggesting the presence of glycogen. Positive PAS staining of azurophils has also been reported in the green iguana, rainbow lizard, and black and white tegu, among others, and is considered characteristic for this cell type (Campbell and Ellis, 2007; Carvalho et al., 2006; Caxton-Martins and Nganwuchu, 1978; Harr et al., 2001). Metachromatic staining of the cytoplasm with toluidine blue indicates the presence of acid polysaccharides. Previous reports of azurophils staining with toluidine blue in other reptile species could not be found.
Although no Sudan black or acid phosphatase stains were performed, bobtail azurophils were not peroxidase positive, which is similar to that of other lizards. This suggests that the function of bobtail azurophils is more similar to mammalian monocytes than neutrophils, as is found with most lizards (Stacy et al., 2011).

Due to the low number of monocytes on the smears, the cytochemical staining characteristics could not be identified. Thus no further conclusions can be drawn with regards to function.

**Lymphocytes**
None of the cytochemical stains applied to the smears stained the cytoplasm of lymphocytes. This is consistent with results reported from most other reptile species (Raskin, 2010).

**Thrombocytes**
The cytoplasm of thrombocytes contained focal granules that were PAS-positive, suggestive of glycogen bodies. A similar staining pattern was seen in thrombocytes of the green iguana, however there appeared to be more positive-staining granules in the iguana thrombocytes than those in the bobtails in this study (Harr et al., 2001). PAS-positive staining of thrombocytes has also been reported in the desert tortoise, eastern diamondback rattlesnake, yellow rat snake and pit viper (Alleman et al., 1992, 1999) (Bounous et al., 1996; Egami and Sasso, 1988). Thus PAS can be used to assist differentiation of individual thrombocytes from lymphocytes in bobtails, and as a quality control method to ensure correct lymphocyte counting.

**Erythrocytes**
The cytoplasm of erythrocytes stained diffusely pale brown with benzidine peroxidase. Haemoglobin has peroxidase-like activity, accounting for its staining (Kiernan, 1999). On smears stained with PAS, there were several small positive-staining inclusions within the
cytoplasm of most mature erythrocytes, considered likely aggregates of glycogen. These aggregates may be associated with degeneration of organelles due to smear preparation (Alleman et al., 1992).

Although only a limited number of cytochemical stains were used in this study, it enabled further characterisation of cellular contents, and the PAS provided a method of differentiation between small lymphocytes and thrombocytes, which was occasionally problematic. Expanding the panel of cytochemical stains to include chloroacetate esterase, alpha-naphthyl butyrate (nonspecific) esterase, Sudan black B, leukocyte alkaline phosphatase and acid phosphatase would allow further investigation of the cytoplasmic constituents of the leukocytes and therefore, a greater understanding of the function of the cells (Raskin, 2010). The cytoplasmic contents of the eosinophil, in particular, require further investigation.

1.3.3 Comparison with the Canfield and Shea (1988) study

Comparing the data from the Canfield and Shea (1988) study to this study yields several points of interest. The red blood cell counts were an average of $0.32 \times 10^{12}/L$ lower in this study, and the white blood cell counts were an average of $3.01 \times 10^9/L$ higher. The mean lymphocyte count was also higher in this study (16.1% compared to 6.3% in the 1988 study), although statistical comparison the leukocyte data was not performed as the definition of the granulocytes differed (see below). Although this is certainly interesting, no interpretation of the validity of either data set can be used as a yardstick for the other for several reasons. The main reason is the difference in preanalytical factors, chiefly geographical location (New South Wales vs Western Australia), housing (wild-caught vs captive), season (not specified in the Canfield and Shea study) and anticoagulant used (K$_2$EDTA vs lithium heparin). These factors are known to potentially have a strong influence on haematological data in reptiles.
There were also differences in analytical factors. The manual cell counts were performed by different methods. The Unopette® dilution was used in the Canfield and Shea study which provides an accurate method of diluting blood samples for cell counts but does not stain the cells. This may result in difficulty differentiating leukocytes from polychromatophils (Campbell, 2004). In this study, Natt & Herrick’s solution was used to dilute and stain blood samples, which utilises methyl violet to stain the cells. Natt & Herrick’s stain solution allows better definition of cellular contents.

There was a difference in the classification of the granulocytes between this study and that of Canfield and Shea. The classifications in this study were made following consultation with Professor Canfield himself, Professor Alleman and Dr. Stacy as previously discussed. The cells identified by Canfield and Shea (1988) as degranulated heterophils were classified as eosinophils in this study. It is difficult to be certain from the small black and white images in the paper, but the cells classified as eosinophils by Canfield and Shea (1988) appeared similar to the granulated, non-vacuolated eosinophils which were seen only rarely in this study.

### 1.3.4 Plasma biochemistry

With biochemistry values obtained from only eight bobtails, no reference intervals can be derived and few conclusions may be drawn from such a small sample size. However, results were less variable between individuals than found with the haematology.

Uric acid was the analyte that seemed to vary the most in the bobtails (mean 0.16mmol/L, SD 0.09mmol/L). The normal plasma uric acid concentration of reptiles is reported as less than 0.6mmol/L (Campbell, 2006). In the green iguana, plasma uric acid concentrations varied from 0.05 to 0.40mmol/L (Harr et al., 2001). All eight bobtails in this study had uric acid concentrations less than 0.6mmol/L, with the highest value 0.31mmol/L. Thus the variability in uric acid concentrations is unlikely to be of any clinical significance.
The normal plasma total calcium concentration for reptiles is reported as 2.2-2.75mmol/L. Hypercalcaemia has been defined as greater than 5mmol/L (Campbell, 2006). Of the eight captive healthy bobtails, seven bobtails had plasma total calcium levels of greater than 2.75mmol/L, with a mean of 2.96mmol/L (SD 0.27mmol/L); the highest value was 3.24mmol/L. In a study of captive green iguanas, adult male iguanas (n=18) had plasma total calcium concentrations of 2.15-3.6mmol/L (mean 2.82mmol/L, SD 0.3mmol/L) (Harr et al., 2001). The plasma total calcium of wild lace monitors (n=33; sexes were not known) was 2.63-3.76mmol/L (mean 3.29mmol/L, SD 0.26mmol/L) (Scheelings and Jessop, 2011). The mean plasma total calcium concentration of captive Gila monsters has been reported as 3.05mmol/L (SD 0.2mmol/L) (n=16) (Cooper-Bailey et al., 2011). Thus with the wide species variations seen, the plasma total calcium concentrations were considered unlikely to be abnormal in these bobtails. Dietary calcium supplements are commonly provided to captive lizards for prevention of metabolic bone disease, and although details of type and frequency of supplements were not collected for the bobtails in this study, supplementation is likely contributing to results. As in mammals, total calcium concentration can be influenced by many factors, especially albumin concentration, thus ionised calcium is considered a better indicator of the biologically active plasma calcium levels (Campbell, 2006). Further studies measuring total and ionised calcium in bobtails (with a larger sample size) would be required to further evaluate.

Campbell (2006) also reported normal plasma glucose concentrations for reptiles. The range given was 3.33-5.55mmol/L. There is a with marked physiological variation between reptile species with glucose concentration, and an influence by ambient temperature and nutrition (Coulson and Hernandez, 1964; Stein, 1996). The mean plasma glucose concentration for the eight bobtails in this study was 6.9mmol/L (SD 1.64mmol/L; range 5.1-9.9mmol/L). As in mammals, catecholamines and corticosteroids can elevate plasma glucose in reptiles, thus these higher results may be normal for this species, due to acute (catecholamine) stress from handling and venepuncture, and/or due to chronic (corticosteroid) stress from captivity (Gregory and Schmid, 2001; Stahl, 2003). Measurement of corticosterone levels would be required to further investigate the effect of stress in captivity.
The mean plasma inorganic phosphate concentration of the eight bobtails was 1.19mmol/L (SD 0.27mmol/L), with a range of 0.9-1.6mmol/L. All values lie within the normal reptilian plasma inorganic phosphate concentration range which was reported as 0.32-1.62mmol/L (Campbell, 2006).

The total protein (biuret) concentrations of the eight bobtails were also within the normal suggested range for reptiles of 30-70g/L (Campbell, 2006). The mean total plasma protein in this study was 49g/L (SD 8.34g/L), with a range of 32-58g/L. The plasma (biuret) total protein was between 1 and 5g/L lower than the total plasma protein as measured by refractometry, likely due to the presence of fibrinogen and other solids which are also detected by refractometry.

The plasma glucose concentration was higher than that reported for other species, with acute and/or chronic stress considered the most likely reason. The plasma total calcium concentration was higher than that stated as normal for most reptiles by Campbell (2006). Several other captive and wild reptile species also had higher plasma total calcium than this reported range and the bobtails in this study were receiving variable dietary calcium supplements, hence this is also likely to be contributing. Although it was not the main focus of this study, clinical biochemistry is also important for the health assessment of reptiles and further studies with a larger sample size are required to generate biochemistry reference intervals for this species. Comparison with wild bobtails would also be useful.

1.3.5 Transmission electron microscopy

The preservation of the cells was adequate for ultrastructural examination and the method of fixation, dehydration and embedding as used for tissues also was suitable for the cell pellets.
The resin did not penetrate completely through the block for some samples, resulting in poor sections, and additional embedding of the block was required. Reducing the aliquot of whole blood used, perhaps to 10 or 20μL would minimise this problem. Removal of the plasma prior to fixation may also be advantageous for preservation. This was not done in this study as samples were collected in the field where a centrifuge was not available.

The ultrastructure of the blood cells was consistent with descriptions of bobtail blood cells by Canfield and Shea (1988) and was generally comparable to those reported for other reptiles (Carvalho et al., 2006; Casal et al., 2007; Salakij et al., 2002; Work et al., 1998; Zhang et al., 2011).

**Eosinophils**
Electron microscopy was required to confirm the identity of the vacuolated cells as eosinophils, as the light microscopic morphology and cytochemical staining panel were equivocal. On light microscopy, it was not clear if the eosinophils had true cytoplasmic vacuoles, or if they were merely non-staining granules as in the green turtle and greyhound (Iazbik and Couto, 2005; Work et al., 1998). Ultrastructural examination of the bobtail eosinophils revealed they were indeed true vacuoles, with the presence of crystalline material at the periphery of each vacuole suggesting that the granules had degranulated or were otherwise degenerate. The reason for this is not clear but as it was a uniform change across all bobtails examined, and this has also been reported in the olive ridley sea turtle, it likely involves sample collection or handling (Zhang et al., 2011).

Vacuoles may be seen in many cell types, but the presence of the crystalline material within the vacuoles confirmed the cell as an eosinophil. This is consistent with descriptions from other reptile species (Carvalho et al., 2006; Work et al., 1998). The eosinophil granules of many mammals contain crystalline material formed by major basic protein. The presence of similar material in the granules of the bobtail lizard may suggest the presence of major basic protein, however immunogold labelling would be required to confirm this (Egesten et al., 1986; Peters, 1986).
The nucleated cells with the large magenta granules were too rare to be identified on TEM sections, however the findings above further support that the circulating form of eosinophil most often seen is degranulated. The reason for degranulation is not known.

Haemogregarine parasites
The haemogregarine gamonts seen in erythrocytes appeared morphologically similar to those of *Hepatozoon* in the king cobra (Salakij et al., 2002). However, the light microscopic morphology of the bobtail haemogregarines does differ from the king cobra *Hepatozoon* which is a larger, sausage-shaped gamont with a central nucleus. The light microscopic features of the haemogregarines were virtually identical to the *Hemolivia mariae* gamonts and merozoites reported previously in the bobtail lizard by Smallridge (1998). The ultrastructure of *Hemolivia mariae* was, however, quite different - an elongated, dumbbell shape was seen in the Smallridge (1998) study compared to the ovoid parasites seen in this study. The Smallridge study involved experimental infections, and there were much higher numbers of intraerythrocytic merozoites (an earlier life stage) than in the bobtails in this study. As well as merozoites, gamonts were also seen by light microscopy by Smallridge and it was stated that the blood samples upon which the TEM was performed contained both circulating merozoites and gamonts. However, it is possible that the electron photomicrographs provided by Smallridge were only of early merozoites and in this study, later merozoites and gamonts were seen. The other possibility is that the haemogregarines in this study are not *Hemolivia* but a different genus. A defining feature of the *Hemolivia* gamont is the presence of suture points within the plasmalemma. Suture points were not identified in the gamonts in this study, however, only five gamonts were seen, and the preservation of them was suboptimal: particularly of the plasmalemma. Examination of gut contents from infected ticks for the star-shaped oocysts which also typify *Hemolivia* species would provide further corroboration (see 2.2.1).

Heterophils
The heterophils of bobtails appear to contain at least two types of granules as in many other reptile species (Carvalho et al., 2006; Salakij et al., 2002). The large, electron-dense, oval to
elongate cytoplasmic granules most likely correspond to the bright eosinophilic granules seen on Wright’s-Giemsa stained blood films.

**Monocytes**
Monocytes were seen with variable numbers of cytoplasmic granules. The monocytes which contained moderate numbers of small granules would be likely consistent with the azurophils seen by light microscopy. The monocytes which had few granules may represent non-azurophilic monocytes or poorly granulated azurophils. This variability of granulation in the monocytic cells is consistent with that reported in other reptile species (Martinez-Silvestre et al., 2005; Salakij et al., 2002). Martinez-Silvestre *et al.* (2005) also used the nuclear to cytoplasmic ratio to ultrastructurally differentiate monocytes from azurophils in the giant lizard but there was not a noticeable difference in the monocytes in this study. However, as monocytes were rare on the smears, it is likely that they were not visualised on the grids at all and all monocytes represented variably-granulated azurophils.

**Thrombocytes**
Bobtail thrombocytes contain a canalicular network, as in some other reptiles, as well as the platelets of many mammals (Casal et al., 2007; Cheville, 2009; Daimon et al., 1987). This provides additional information on the function of bobtail thrombocytes, indicating that they release granules and other materials into the canalicular system for rapid release from the cell, rather than by exocytosis (Cheville, 2009).

**Lymphocytes, erythrocytes and basophils**
The ultrastructural morphology of lymphocytes and erythrocytes were consistent with those reported in other reptile blood cell ultrastructural studies (Casal et al., 2007; Salakij et al., 2002; Work et al., 1998; Zhang et al., 2011).

Unfortunately no basophils could be identified under the transmission electron microscope. Basophils accounted for less than 8% of the leukocyte differential count in all the samples.
where cell blocks were prepared, so they were too few in number to be seen. Further studies examining ultrathin sections of the buffy coat of the blood sample would be required to increase leukocyte yield and enable identification of basophils.

The cytochemistry and ultrastructure of the leukocytes yielded further information about their structure and function. The granules of heterophils were peroxidase-positive, indicating that they contain myeloperoxidase and are therefore most likely capable of production of free radicals during inflammation. The vacuoles of the eosinophils contained crystalline material, suggestive of major basic protein. Major basic protein is a potent cytotoxic protein to organisms and host cells which is also involved in inflammation in mammals (Young and Meadows, 2010). It is likely that the same is true in reptiles. Thrombocytes contain a canalicular structure which enables rapid release of cellular contents from the cell following appropriate stimulation.

1.3.6 Blood collection

As successful venepuncture was sometimes difficult, and the technique had not been reported elsewhere, a brief discussion of the difficulties encountered and the optimal method of blood collection follows.

The venepuncture technique was very well-tolerated by the vast majority of lizards, although it was often slow. Most lizards remained still during collection, and additional restraint was not generally required, although placement of a hand over the upper half of the lizard by an assistant was advantageous for some collections. Typically the first venepuncture site accessed was caudal, approximately three quarters of the length of the tail, and if an additional sample site was required, it would be more cranial. The ventral coccyygeal vein is thinner caudally and blood flow is slower, but there is less subcutaneous fat.
Venepuncture was more difficult in the bobtails with more fat in their tails. Fat blockage of the needle was encountered frequently when redirection was required so replacement of the needle was best if the vein was not entered after a few redirects. Thicker tails required longer needles and this also decreased the precision of the redirections. For most healthy bobtails, the 23G, 1 inch needle was optimal. The lack of correlation between tail thickness and blood volume obtained \((r=0.076)\) suggested that although blood collection often took longer in the lizards with thicker tails, this did not impact the final volume of sample obtained.

Lymph flow was noted on several occasions during venepuncture. The lymph flowed on its own and did not mix with venous blood. In these instances, the needle and syringe was discarded and a new site was accessed.

Lithium heparin was used as the anticoagulant in this study as EDTA causes lysis of the blood cells of some reptiles and biochemical analysis of heparinised plasma could also be performed (Strik et al., 2007). Clotting of the sample did not appear to be a problem in this study, with only one smear containing clots. This smear was one made from only a very small volume of blood (~0.03mls). It is likely that the blood volume collected was insufficient for the sample to contact the disc of lithium heparin anticoagulant.

Thus the venepuncture technique used in this study was well-tolerated by bobtails and generally successful to yield a blood sample of sufficient volume for haematological analysis.

**1.3.7 Bobtail morphometrics**

The mean snout to vent length of the lizards (277mm) was very near the average of 280mm reported by Bull (1995). Thus, despite the difference in geography from the previous study (based in South Australia), and the fact that Bull was measuring wild adult bobtails, not captive bobtails as in this study, this data affirms this mean adult length and can be used to
assess maturity in this species. Male and female bobtails reportedly have the same mean SVL (Bull and Pamula, 1996).

The SVL of one lizard was greater than three standard deviations below the mean (225mm), suggesting that this bobtail may have been immature, or possibly did not attain normal body size due to disease or poor diet. This small bobtail appeared clinically healthy and was kept in the same enclosure and fed the same diet as several other lizards of more normal size which were also sampled in this study. No information was available about length of time in captivity for this lizard. Despite its possible status as immature, there were minimal differences in the haematology. When this bobtail (Healthy 9) was excluded from the population and the Reference Value Advisor v2.1 macro for Microsoft® Excel® (National Veterinary School of Toulouse, Toulouse, France) was ran again for all data, the means were all similar. The upper reference interval of the white blood cell count was slightly higher at $38.9 \times 10^9/L$, compared to the original of $31.0 \times 10^9/L$. As the reference interval for white blood cell count was so wide, this was not considered a clinically significant change and thus this bobtail was not removed from the sample population.
1.4 Summary of the haematology of healthy bobtail lizards

This study has produced reference intervals for the haematology of healthy, captive, adult *Tiliqua rugosa* in summer in Western Australia. The morphology of blood cells as examined by both light microscopy and transmission electron microscopy has been described. The presence of almost uniformly degranulated eosinophils on the smears was a finding not previously reported in reptiles. Intraerythrocytic haemogregarine parasites were identified on the smears from several lizards, with evidence of a pathological response to infection due to an associated decrease in PCV and increase in polychromasia in infected lizards. These changes may reflect haemolysis due to infection. Cytochemical stains were used to assist characterisation of cellular contents and therefore function of the blood cells, indicating the role of the heterophil in oxidative killing and the cytotoxicity of the eosinophil to organisms. A very small study on the plasma biochemistry of bobtails has been described, paving the way for future work.
CHAPTER 2: PATHOLOGICAL CHANGES IN BOBTAIL LIZARDS AND
THE TICKS WHICH PARASITISE THEM

2.1 Bobtails with upper respiratory tract disease

Kanyana Wildlife Rehabilitation Centre is one of the largest wildlife rehabilitation organisations in Western Australia. Sick or injured bobtail lizards are commonly brought in by members of the public to receive care. One of the most common reasons for presentation is upper respiratory tract disease, with Kanyana admitting approximately 120 bobtails each year, with the majority in the late spring to early autumn (Haight, 2013).

The reference intervals, cytological findings and data developed in the study of healthy bobtails were used to assess bobtails with upper respiratory tract disease.

2.1.1 Materials and methods

Over the summer of 2012/2013, blood samples were collected from seven adult bobtails hospitalised for upper respiratory tract disease (URTD). All bobtails were kept in temperature-controlled vivaria. Only bobtails which were not obviously pregnant or moribund were included. Six bobtails from Kanyana Wildlife Rehabilitation Centre and one bobtail from Murdoch University Veterinary Hospital (MUVH) met the selection criteria. For each bobtail, a history was obtained, an examination was performed, morphometric measurements were taken, and a heparinised blood sample was collected and processed as per 1.1 Materials and methods. In addition, the severity of clinical signs was assessed subjectively and minimal, mild, moderate or severe. Clinical signs classified as minimal included a bright and alert demeanour, pink mucous membranes, a very small amount of
serous ocular, nasal or choanal discharge, occasional sneezing, and a body condition score of greater than 2.5 out of 5. Mild signs were a slightly depressed demeanour, slightly pale pink mucous membranes, a small amount of serous ocular, nasal or choanal discharge, and a body condition score of 2.5 out of 5). Moderate signs were typified by a depressed demeanour, pale pink mucous membranes, a moderate amount of serous ocular, nasal or choanal discharge, and a body condition score of 2 out of 5. Bobtails which would have been classified with severe clinical signs (depressed, white to pale pink mucous membranes, profuse discharge, and body condition score less than 2 out of 5) were not sampled to avoid causing further morbidity, as per Kanyana’s request.

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8 Murdoch University Veterinary Hospital, Murdoch drive, Murdoch, Perth, Western Australia 6150, +61893602494

The tail thickness was compared to the healthy data using an independent t-test. Just as for the healthy bobtail data, significance was set at P<0.05.

2.1.2 Results

Data was collected on the history, morphometry, disease severity and blood volume collected (Table 12). Bobtails were referred to as URTD 1 to 7. The mean snout to vent length was 280mm, mean tail thickness was 28mm and the mean weight was 443g.
Table 12: Upper respiratory tract disease bobtail population data (eod= every other day, viv= vivarium, ID= identification).

<table>
<thead>
<tr>
<th>URTD #</th>
<th>Source</th>
<th>ID</th>
<th>Date collected</th>
<th>Admission date</th>
<th>Faecal egg count results</th>
<th>Medications received</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MUVH</td>
<td>n/a</td>
<td>12/12/2012</td>
<td>12/12/2012</td>
<td>Not performed</td>
<td>Intramuscular meloxicam and enrofloxacin injections. Intraperitoneal fluids one hour before blood collection.</td>
</tr>
<tr>
<td>2</td>
<td>Kanyana</td>
<td>17286</td>
<td>3/01/2013</td>
<td>30/12/2012</td>
<td>Trematodes, Trichomonas, Balantidium</td>
<td>Enrofloxacin injections eod, metronidazole and fenbendazole oral (parasites) Also had healed tail tip wounds.</td>
</tr>
<tr>
<td>4</td>
<td>Kanyana</td>
<td>17364</td>
<td>9/01/2013</td>
<td>5/01/2013</td>
<td>Not performed</td>
<td>Enrofloxacin injections eod.</td>
</tr>
<tr>
<td>5</td>
<td>Kanyana</td>
<td>17346</td>
<td>9/01/2013</td>
<td>4/01/2013</td>
<td>Not performed</td>
<td>Enrofloxacin injections eod. Tricin eye ointment.</td>
</tr>
<tr>
<td>6</td>
<td>Kanyana</td>
<td>17422</td>
<td>16/01/2013</td>
<td>14/01/2013</td>
<td>Not performed</td>
<td>Enrofloxacin injections eod. Tricin (polymixin B, bacitracin and neomycin) eye ointment.</td>
</tr>
<tr>
<td>7</td>
<td>Kanyana</td>
<td>17643</td>
<td>26/02/2013</td>
<td>28/02/2013</td>
<td>Not performed</td>
<td>Enrofloxacin injections eod</td>
</tr>
</tbody>
</table>

Medications had been administered to all bobtails prior to blood collection. Faecal egg counts were only performed for two bobtails. Blood was collected from bobtails that had been hospitalised for as little as one hour (URTD 1) but up to a total of 11 days (URTD 4). The vivariums were all set at 28-30°C, just below preferred body temperature of 34°C.

There was no significant difference between the tail thickness of the upper respiratory tract disease bobtails and the healthy bobtails (P=0.108).

Complete blood counts and Wright’s-Giemsa stained blood film evaluations were performed (Table 13).
Table 13: Complete blood count data for upper respiratory tract disease bobtails (insufficient= insufficient blood volume to perform the test). Highlighted in red are those results outside the healthy bobtail reference intervals.

<table>
<thead>
<tr>
<th>URTD #</th>
<th>PCV (L/L)</th>
<th>Total plasma protein (g/L)</th>
<th>RBC Count (10^12/L)</th>
<th>WBC Count (10^9/L)</th>
<th>Hb (g/L)</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>MCHC (g/L)</th>
<th>Polychromatophils (per 100 rbcs)</th>
<th>Degree of polychromasia</th>
<th>Haemogregarines (per 10,000 rbcs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.32</td>
<td>64</td>
<td>insuff</td>
<td>insuff</td>
<td>insuff</td>
<td>insuff</td>
<td>insuff</td>
<td>insuff</td>
<td>4.8</td>
<td>Slight</td>
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<td>128</td>
<td>302</td>
<td>1.2</td>
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<th>URTD #</th>
<th>Heterophils</th>
<th>Immature heterophils</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
<th>Eosinophils</th>
<th>Basophils</th>
<th>Reactive lymphocytes (per 100 wbc)</th>
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<td>1</td>
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<td>10</td>
<td>11</td>
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<td>1</td>
</tr>
<tr>
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<td>27.5</td>
<td>7</td>
<td>39.5</td>
<td>17</td>
<td>7</td>
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<td>37.5</td>
<td>11</td>
<td>10.5</td>
<td>3</td>
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Bobtail URTD 2 had a PCV and total plasma protein below the healthy reference intervals of 0.10-0.44L/L and 36-74g/L respectively. This bobtail also had a slightly higher polychromatophil count (reference interval 0-7%). Immature heterophils were seen on the smears of four lizards (see below); immature heterophils were not identified on any smears from healthy bobtails. Lymphocyte percentages of five URTD bobtails were higher than the healthy lizards (reference interval 0-34%). The monocyte percentage was higher for URTD 1 (reference interval 1-27%).

For two bobtails, sufficient plasma remained to run a panel of plasma biochemistry analytes (Table 14).

Table 14: Heparinised plasma biochemistry results for bobtails 3 and 4 with upper respiratory tract disease.

<table>
<thead>
<tr>
<th>URTD #</th>
<th>Uric acid (mmol/L)</th>
<th>Total protein (g/L)</th>
<th>Albumin (g/L)</th>
<th>Globulins (calculated)</th>
<th>Inorganic phosphate (mmol/L)</th>
<th>Calcium (total) (mmol/L)</th>
<th>Glucose (mmol/L)</th>
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<td>3</td>
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<td>47</td>
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<td>30</td>
<td>1.3</td>
<td>3.12</td>
<td>8.5</td>
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</table>

Although only two samples were analysed, results were similar for most analytes. The glucose and more so, the total calcium, did vary somewhat. All results fell within the range of results found in the healthy bobtails.

On the Wright’s-Giemsa-stained blood smears from the URTD bobtails, no granulated eosinophils were seen but all the other blood cells seen on smears from healthy lizards were present and generally morphologically identical. However, in four of the bobtails (URTD 1 to 4), there was a left shift in the heterophils (Figure 60). Immature heterophils had a bilobed or ovoid nucleus with often a paler, more open chromatin pattern, a pale eosinophilic cytoplasm - paler than that seen in mature heterophils - and low numbers (1-15) of bright purple (amphophilic), round granules. In the heterophils from URTD1, there were 2+ to 3+ toxic changes present, with decreased granularity, increased cytoplasmic basophilia and mild cytoplasmic vacuolation (Figure 60).
Figure 60: Immature heterophils with few round, purple granules (arrows). The immature heterophils in the centre image and on the right have mild to marked toxic changes respectively, with enhanced cytoplasmic basophilia and cytoplasmic vacuolation. (1000x magnification, Wright’s-Giemsa)

In URTD 1, changes were also noted in the azurophils. There was a decrease in granularity in many of the azurophils with often a paler, more open chromatin pattern. Very rare binucleate azurophils were also seen (Figure 61).

Figure 61: Azurophils in URTD1 blood smear. Left- An immature heterophil (left) with mild cytoplasmic basophilia and vacuolation (mild to moderate toxic changes), and an azurophil (right) with open chromatin and minimal granularity. Centre- An azurophil with open chromatin and the cytoplasm contains a moderate decrease in granularity. Right- A binucleate azurophil (1000x magnification, Wright’s-Giemsa)

2.1.3 Discussion

It was originally anticipated that approximately 40 bobtails with upper respiratory tract signs would be available for blood sampling over the two summer sampling periods. Unfortunately there was a delay with approval from the main wildlife rehabilitation centre, Kanyana, which meant that no collections could occur over summer 2011/2012. There were low numbers of sick bobtails available over the summer of 2012/2013, with only seven
meeting the inclusion criteria. It is not known if the disease is decreasing in prevalence, there were seasonal changes, if more sick bobtails were admitted in the months before December or after February, or if the reason for the decrease in submissions was attributable to fewer members of the public observing or admitting sick lizards. Due to the low number of samples, no concrete conclusions can be drawn from the data, but some key observations were made which requires further verification with a larger sample size.

One of the largest drawbacks of this short study was the fact that the samples were all collected after treatment had been administered. Pre-treatment sampling was not possible as the priority of the rehabilitation centres is to administer treatment. Thus it is not known if the haematology of these lizards differs at admission. It must also be considered that the duration of illness for each lizard prior to admission was not known.

The URTD 1 bobtail was sampled only one hour following treatment, and this bobtail had the most marked changes on the leukon. It had a mild left shift, as did URTD 2, 3 and 4, which was typified by heterophils with round, bright purple granules (primary granules) in the cytoplasm (Strik et al., 2007). URTD 1 also had toxic changes evident in the heterophils, with particularly notable cytoplasmic basophilia. There was also an increased percentage of monocytes (41.5% compared to the reference interval of 1-27%) with changes in the morphology of some azurophils. The changes were decreased granularity and a more open chromatin pattern, likely reflecting a left shift in this cell line. Thus the left shift in heterophils and possibly azurophils suggests increased tissue demand for these leukocytes, suggestive of inflammation and likely bacterial infection in this lizard (Strik et al., 2007). The left shift seen in the three other lizards was also suggestive of inflammation; however the degree of the left shift and lack of concurrent toxic changes is less suggestive of overt bacterial infection in these bobtails. Again, whether this is representative of all bobtails with upper respiratory tract disease cannot be confirmed at this early stage of investigation. There is a paucity of published information about the haematological response to respiratory diseases in other reptiles, with the exception of desert tortoises with *Mycoplasma agassizii* infection in which a non-regenerative anaemia was the only abnormality (Jacobson et al., 1991). Thus it is difficult to appreciate how frequently a left shift is seen during respiratory disease in reptiles, and with which aetiological agents.
Comparing the haematological results to the reference intervals (with 90% confidence intervals) developed in Chapter 1 of this study, lizard URTD 2 had a mildly regenerative anaemia and a hypoproteinaemia. This may have been attributable to the intestinal parasites detected by the faecal egg float. The tail tip wounds are another possibility but these lesions appeared to represent historical trauma, and it was considered unlikely that the regenerative response of the erythrocytes would have still been ongoing.

There was an increased percentage of lymphocytes (0-34% reference interval) in URTD 2, 3, 4, 5 and 7 ranging from 30.5% to 50.5%. An increase in lymphocytes may be due to inflammation, wound healing, parasitic infections, or viral diseases (Campbell, 2006). URTD 2 and 3 both had intestinal parasites, and a faecal float was not performed for URTD 4, 5 and 7, thus intestinal parasitism is one possible cause. The other likely cause is the upper respiratory tract disease itself. Reactive lymphocytes were not a prominent feature on the blood films of any URTD lizards, thus it may be considered that the increase in percentage lymphocytes may be a subacute or acute change rather than a chronic change.

The plasma biochemistry results for URTD 3 and 4 were very similar to those of healthy bobtails. Both lizards had slightly higher plasma glucose concentrations of 7.6 and 8.5mmol/L than reported by Campbell (2006) as normal for reptiles (3.33-5.55mmol/L), but within the range of the healthy bobtails. Again, the elevated blood glucose concentrations may be due to an acute catecholamine stress response, a corticosteroid-related change, or may be normal for this species. The plasma total calcium concentration of URTD 4 was also mildly increased above the normal of 2-2.75mmol/L, at 3.12mmol/L. This was still within the range seen in the healthy bobtails. As discussed in the healthy bobtails, many intrinsic and extrinsic preanalytical factors influence total calcium concentration, and as such, this was considered unlikely to be clinically significant.

When compared to the reference intervals constructed in the healthy bobtails and in the light microscopic morphological features, there were changes in the haematology of all lizards except URTD 6 which suggested inflammation. Anti-inflammatory drugs may therefore be useful in the treatment of this disease. Thus, despite the wide 90% confidence intervals, the reference intervals appear to be clinically useful. The main caveat to this is
that the intervals were constructed from bobtails which had been in long term captivity, and any differences in the haematology between captive lizards, wild lizards, and recently captured lizards are currently unknown.

No haematological changes that were seen were considered to be specific for a particular infectious aetiological agent. The interpretation of results was further complicated by intestinal parasitism, of which the clinical significance is not known. The left shift and toxic changes seen may suggest a bacterial aetiology, but secondary bacterial infections are extremely common in respiratory tract disease due to other infectious and non-infectious aetiologies (Schumacher, 1997). That said, the results of this study do suggest that the inclusion of antibiotics in the treatment regime of bobtails presenting with upper respiratory tract signs is also likely of benefit and should continue.

To the author’s knowledge, there are no published studies on morphometric measures of morbidity in reptiles. Tail thickness, as measured immediately caudal to the pelvis, was hoped to be a novel measure of morbidity in the bobtails. This location was used as tail lengths vary between lizards, so this site was standard. Certainly subjectively, many lizards admitted with upper respiratory tract signs have a prominent pelvis and sometimes even the dorsal processes of the coccygeal vertebrae can be seen. The mean tail thickness was not significantly different from the healthy bobtails and thus this was not a useful measure of morbidity. This was thought to be an issue with the location of measurement, which really reflects pelvis height rather than the tail itself. Specifying a distance of a certain number of scales caudal from the cloaca may be a better method of standardising the measurement of tail thickness and therefore the amount of fat present, as a reflection of body condition.

The other attempt at characterising the degree of morbidity was the subjective scale of severity based on pallor of mucous membranes, amount of nasal discharge, and body condition score. There did not appear to be a correlation with this score and the haematological changes. Whether or not it is an indication of rehabilitation time or release success is not known, so at this stage, further application of this scale is not recommended.
2.2 Additional findings

During this study, several opportunities arose to further investigate aspects of bobtail haematology which were outside the original aims of the thesis but provided valuable supporting information. Some bobtails in this study were parasitised by ticks. Several of the ticks were collected and cytology and histopathology were performed to search for haemogregarine parasites. Archived histopathology slides from bobtails with and without upper respiratory tract disease were reviewed. Finally, a bobtail with multiple traumatic skull fractures was admitted to Murdoch University Veterinary Hospital and subsequently euthanased and submitted for post mortem. A very short post mortem interval enabled the collection of well-preserved bone marrow for cytological and histopathological examination.

2.2.1 Histopathology and cytology of ticks

Several of the healthy bobtails had hard-bodied ticks present within the external auditory meatuses (Figure 62).

Figure 62: Hard-bodied ticks (*Amblyomma albolimbatum*) collected from several bobtails.
Three ticks from different lizards were removed and fixed in glutaraldehyde. The collected ticks were identified by the Parasitology Department at Murdoch University as *Amblyomma albolimbatum*. *Amblyomma albolimbatum* is known as the stump-tailed lizard tick, and is one of three species of ixodid ticks commonly found on bobtails (Myers et al., 2013). The ticks were processed for histology, serially sectioned and stained with haematoxylin and eosin (H&E). Smears were made from the abdominal contents from two additional, non-fixed ticks, and air-dried and stained with Hematek® Wright’s-Giemsa stain (Siemens Healthcare, Victoria, Australia).

Histology of the ticks revealed the presence of protozoal parasites within the gut epithelium, and occasionally, free in the lumen. The majority of the parasites were seen as cysts (meronts) which contained 7-16 elongate merozoites approximately 6µm long and 1µm wide. The merozoites had a small, round, basophilic nucleus and a homogenous, eosinophilic cytoplasm (Figure 63). The meronts were within the cytoplasm of epithelial cells and occasionally pushed the nucleus to one side. A single ovoid zygote of approximately 23µm diameter with a dense, small central nucleus was seen within the lumen of the gut on one section (Figure 64).
Figure 63: Histology of a tick with meronts (arrows) within the epithelium of the gut (L=lumen). (Top left 100x, top right 400x and bottom 1000x magnification, H&E)

Figure 64: Histology of a tick with a protozoal zygote (arrows) within the gut lumen. (400x and 1000x magnification, H&E)
These meronts were of a similar morphology to those reported previously in *Amblyomma limbatum* ticks which were placed on bobtail lizards experimentally infected with *Hemolivia mariae* (Smallridge, 1998). Speciation of protozoal cysts and zoites on the basis of light microscopy is not possible. A unique feature of both *Hemolivia mariae* and *Hemolivia stellata* (the latter infecting the toad *Bufo marinus*) is the formation of star-shaped oocysts in the epithelium of the gut of the tick host (Petit et al., 1990; Smallridge, 1998; Telford Jr., 2008). A single star-shaped oocyst was found in one of the tick abdominal content smears, confirming the presence of a *Hemolivia* species in the ticks in this study (Figure 65). This is therefore highly suggestive that the haemogregarines seen in the bobtails are also *Hemolivia* species.

![Figure 65: A 3-armed stellate oocyst in an abdominal smear from the tick *Amblyomma albolimbatum*. (200x magnification, Wright’s-Giemsa)](image)

Thus this provides support for the ticks as the source of haemogregarine infection of the bobtails in this study, similar to that reported by Smallridge (1998). Unfortunately there are no molecular tests currently available for detection of *Hemolivia* to confirm the protozoan as of this genus. Studies to correlate the parasitised ticks with the presence of intraerythrocytic haemogregarine gamonts in their bobtail hosts are required to further investigate the lifecycle of this haemogregarine parasite in this tick species.

### 2.2.2 Archived histology sections

Archived histology sections from 2004 to 2013 were retrieved from bobtails submitted to the Pathology Department at Murdoch University Veterinary Hospital. There were ten cases
in total and the reasons for submission were trauma, dystocia, star-gazing, as well as “flu” (i.e., upper respiratory tract disease). Sections from a case of “flu” in a boabtail from the Department of Agriculture and Food Western Australia were also included. As it is currently not known whether there is a tissue site of haemogregarine replication (merogony) in infected bobtails, all sections were also searched for protozoa. Expanding on work already completed by de Poister et al. (2011) (unpublished), sections from bobtails which were clinically diagnosed with “flu” were also examined.

Three protozoal cysts were present in the kidney section from one of the upper respiratory tract disease bobtails (Figure 66). Two cysts were seen within the lumen of a tubule, and the third cyst was located within the cytoplasm of a tubular epithelial cell. The cysts were round and of 20-40µm in diameter with a thin capsule surrounding 50-200 zoites of approximately 2µm in diameter. Zoites appeared round to ovoid but it was difficult to truly discern their shape. The zoites had a round, pale basophilic nucleus with few small chromatin clumps and a pale eosinophilic cytoplasm with indistinct margins. There was no associated inflammation or tissue response.

![Figure 66: Intratubular (left; 400x magnification) and intraepithelial (right; 1000x magnification) apicomplexan protozoal cysts within the kidney section of a boabtail. (H&E)](image)

It was not possible to further identify the protozoan based on morphology alone. This may represent the tissue phase of a haemogregarine such as *Hemolivia*, or it may be another family of protozoa. No blood smears were available for examination from this boabtail. It is concluded that when examining histology sections from bobtails, kidney sections should be carefully examined for meronts.
Archived sections from four bobtails with upper respiratory tract disease were also reviewed. One bobtail had a heterophilic rhinitis and bronchopneumonia, suggestive of bacterial infection, but bacteria were not identified. Another bobtail had a pyogranulomatous keratoconjunctivitis with intralesional degenerate bacterial colonies. There was no evidence of pneumonia or conjunctivitis in the other bobtails, and no nasal or sinus sections were available from these bobtails for examination. No viral inclusion bodies or microorganisms were detected in the nasal section or lung sections from any of the four bobtails.

Thus there were no consistent lesions seen in affected bobtails, but two bobtails had heterophilic inflammation and bacteria were identified in one case (keratoconjunctivitis) by histopathology. As primary bacterial infections of the respiratory tract of immunocompetent reptiles are uncommon, these findings suggest that opportunistic bacterial infections indeed can occur in this upper respiratory tract disease. This is consistent with the left shift and toxic changes considered to reflect bacterial infection in the bobtail URTD 1.

2.2.3 Case study: Bobtail bone marrow cytology and histology

A bobtail was presented to the Murdoch University Veterinary Hospital with multiple skull fractures and subsequent myiasis. Due to the extent of the injuries, the bobtail was humanely euthanased with intravenous pentobarbital and submitted immediately for post mortem. As the carcase was not autolysed, haematopoietic tissues were specifically examined to identify whether eosinophils could be found, and if the blood cell morphology on H&E-stained histology sections was the same as on the blood smears. Bone marrow aspirates were taken using a 23G ¾ inch needle on a 3ml syringe (BD, Singapore, Singapore). The needle was rinsed with K$_2$EDTA. The medullary cavities of the left and right femur and pelvis were aspirated following removal of the metaphysis to expose the marrow cavity. A small amount of material was obtained. Squash preparations were made, then slides were air-dried and stained with Wright’s-Giemsa (Siemens Healthcare, Victoria, Australia). Bone marrow was also submitted for histology. Rib and hemi-pelvis sections were taken, fixed in
buffered formalin for 72 hours, decalcified in 5% nitric acid for 24 hours, sectioned and stained with H&E. Spleen and liver biopsies were also taken for histopathology.

On the cytology and histology of the bone marrow, the majority of the cells seen were developing heterophils. Heterophils were typically present peripherally within the medulla on histology sections. Myelocyte, metamyelocyte and band heterophils (those with primary granules) were prominent (Figure 67).

Admixed with the heterophils were developing eosinophils. The eosinophils on the bone marrow aspirate cytology appeared identical to those seen in the peripheral blood, with a vacuolated appearance. Thus, despite the use of K$_2$EDTA anticoagulant for bone marrow cytology, the vacuolation was persistent, further demonstrating that this change is not attributable to anticoagulant use or type. No granulated eosinophils (with the large magenta granules) were seen.

On the H&E-stained sections of bone marrow, leukocytes considered compatible with eosinophils were seen amongst developing myeloid cells. Eosinophils possessed an eccentric nucleus with clumped chromatin and a cytoplasm with very pale pink round granules (Figure 68). The granules were approximately the same size as the primary and secondary granules in the heterophils. Immature cells also possessed few, dark purple granules (primary granules). Overt vacuoles were not identified.
Figure 67: Bone marrow aspirate cytology photomicrographs with progranulocyte (PG), myelocyte (MC), metamyelocyte (MMC), band (B), mature heterophil (H), polychromatophil (P), eosinophil (E) and rubriblast (RB). (1000x magnification, Wright’s-Giemsa)

Figure 68: Bone marrow histology section from the pelvis with predominantly developing heterophils (thin arrow) and admixed eosinophils (thick arrow). Scattered small mononuclear cells also seen (*). (1000x magnification, H&E)

Basophils were not identified on the H&E, toluidine blue or Giemsa-stained histology sections, and were very rare in the aspirate smears. There were scattered very small clusters of developing azurophils (Figure 69). Also present were low numbers of small mononuclear cells with high nuclear to cytoplasmic ratios which tended to be peripheral in the bone
marrow sections. On histological sections in particular, it was difficult to classify these cells as lymphocytes or thrombocytes, but most were more consistent with lymphocytes (more basophilic cytoplasm). Macrophages were rare, and cytophagia was infrequently noted. There were also moderate numbers of small erythroid islands composed of various stages of developing erythroid cells (Figure 69 and 70). Erythroid islands tended to be more central within the medulla and appeared to be centred on small blood vessels.

Figure 69: Bone marrow aspirate cytology. Left- An immature azurophil (IA), mature azurophil (A), lymphocytes (L), heterophil (H), polychromatophil (P) and band heterophils (B). Right- An erythroid island with various stages of polychromatophilic erythrocytes and an azurophil (A). (1000x magnification, Wright’s-Giemsa)

Figure 70: Histological section of bone marrow with an erythroid island displaying various stages of polychromatophilic erythrocytes and few admixed heterophils. (1000x magnification, H&E)
Occasional haemogregarine gamonts were seen in erythrocytes on the bone marrow aspirate smears.

Adipose tissue comprised approximately 50 to 90% of the medulla on the rib and pelvis histological sections respectively. Rare spindle cells associated with fine fibrous stroma were also scattered throughout the bone marrow on histological sections.

On histological sections, the pelvis marrow was more cellular than the rib marrow. Aspirate cytology from the femur was more cellular and better preserved than the pelvis. The femur is the recommended site of collection in lizards (Campbell and Ellis, 2007). Compared to the (less well-preserved) archived sections of bobtail bone marrow examined, the bone marrow from this bobtail was hypercellular with myeloid hyperplasia of the heterophil and eosinophil lines and possibly the azurophils. This would be consistent with the inflammation secondary to the skull fractures and myiasis.

No overt extramedullary haematopoiesis was seen in the spleen or liver sections, nor those examined from archived tissues.

Assessment of bone marrow from healthy lizards is required to evaluate the normal myeloid to erythroid ratio and assess the relative numbers of each subtype. There is a paucity of information published on the normal bone marrow of reptiles, with most studies limited to discussion of developing cell morphology or the use of cytochemical stains to differentiate the leukocytes (Campbell and Ellis, 2007; Garner et al., 1996). Therefore, further conclusions cannot be drawn from this brief investigation. Special stains such as PAS or immunohistochemistry such as CD4 and CD8 may be required to confidently differentiate thrombocytes from lymphocytes on bone marrow histological sections.
2.3 Summary of the pathological changes of bobtails and the ticks which parasitise them

Despite their abundance in the wild in Western Australia, no published studies on the haematology in disease of *Tiliqua rugosa* currently exist. These short studies represent initial characterisation of the haematological response to upper respiratory tract disease with identified evidence of an inflammatory response, an investigation into the variable and nonspecific histopathological changes in upper respiratory tract disease, and examination of cytology and histopathology of the bone marrow from a bobtail with inflammation.

To the author’s knowledge, this is the first time that some features of haemogregarine infection in bobtails have been identified. Meronts were identified within renal tubular epithelial cells and within the tubular lumen which suggests this as a location of tissue merogony of a protozoal parasite, possibly a haemogregarine. Protozoal meronts have been identified within the gut epithelium of the tick *Amblyomma albolimbatum*. A *Hemolivia* oocyst has been identified in a gut smear of an *Amblyomma albolimbatum* tick parasitising a bobtail. More work is needed to confirm and further elaborate on these findings.
**Final Conclusions**

This study has described the haematology of captive, clinically healthy bobtail lizards (*Tiliqua rugosa*) during the summer months in Western Australia and produced reference intervals for the complete blood count. These reference intervals include 90% confidence intervals such that they include a degree of uncertainty, reflecting physiological variation, and thereby increase their clinical utility.

The morphology of the eosinophils in this species is unique. The vacuolated cytoplasm is a feature seen in the blood smears and bone marrow in this study; and also in the Smallridge (1998) study, and possibly in the Canfield and Shea (1988) study (described as degranulated heterophils on blood smears but as eosinophils ultrastructurally). Very rare cells with large magenta granules were considered to represent the granulated form of eosinophil. The trigger for degranulation is unknown but not attributable to the use of or type of anticoagulant. More work is required to investigate the function of these cells and the significance of the cytoplasmic vacuolation.

Preliminary investigation has been conducted into the clinical chemistry and bone marrow structure and composition of bobtails, paving the way for future studies.

Haemogregarine parasites have been described in the erythrocytes of *Tiliqua rugosa* which appear similar to those previously reported in *Tiliqua rugosa* from South Australia. There is a statistically significant effect of the presence of parasites on the packed cell volume, and a correlation between parasites and polychromasia, suggesting that they may cause some erythrocyte damage or destruction, and may not be non-pathogenic as haemogregarines found in other reptiles are considered to be.

A *Hemolivia* species of haemogregarine parasite has been described for the first time in the stump-tailed lizard tick *Amblyomma albolimbatum* in Western Australia. This suggests that the meronts seen within the tick gut, and the gamonts and merozoites in the bobtail
erythrocytes are also Hemolivia, however more work is required to investigate this hypothesis. The renal tubules are a possible site of tissue merogony in the bobtail host.

Finally, some initial evaluation of the haematology from bobtails with upper respiratory tract disease was performed, utilising the reference intervals developed herein. There was haematological evidence of inflammation in most samples. Following rehydration, there may be a role for non-steroidal anti-inflammatories in the treatment of this disease. One bobtail had a left shift and toxic changes, considered haematological evidence of bacterial infection, and from the examination of archived tissues, bacterial infection was confirmed in one bobtail with upper respiratory disease, and suspected but a causative agent was not detected in one other. Thus the aetiological agent responsible for the upper respiratory tract disease remains elusive, but the presumed secondary bacterial infections seen in some bobtails supports the continuing inclusion of antibiotics in the treatment regime of this disease. Analysis of blood samples from larger numbers of lizards with upper respiratory tract disease is required to assess the significance of these findings, and to ascertain whether haematological evidence of inflammation may be supportive of a diagnosis of upper respiratory tract disease.
REFERENCES


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## APPENDIX: BLOOD CELL MEASUREMENTS

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Erythrocyte measurements from healthy bobtail lizards.
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Range 12-14 9-17 3-4 0.3-0.8 20-90
Mean 13 12 3 0.48 66
Median 13 11 3 0.5 70
SD 0.7 2.8 0.4 0.2 22.6

Heterophils

Heterophil measurements from healthy bobtail lizards.

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N 8 6 6 8
Range 7-17 4-8 4-6 0.4-0.6
Mean 11 6 5 0.5
Median 10 6 5 0.5
SD 3.1 1.4 0.9 2.5

Basophils (µm)

Basophil measurements from healthy bobtail lizards.
### Eosinophils (µm)

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### Azurophils (µm)

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Eosinophil measurements from healthy bobtail lizards.

Azurophil measurements from healthy bobtail lizards.
Monocyte measurements from healthy bobtail lizards.

<table>
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N 5 5 5

Range 9-12 9-11 5-6

Mean 11 10 5

Median 11 10.5 5

SD 2.8 2.3 0.4

Lymphocyte measurements from healthy bobtail lizards.

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N 6 6 6 6

Range 7-9 5-10 10-13 6-10

Mean 8 7 12 7

Median 8 7 12 7

SD 1.2 1.8 2.4 1.5
### Thrombocytes (µm)

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Thrombocyte measurements from healthy bobtail lizards.