

Blood cell histology of *Homopus areolatus*; effects of season and cohort

Sharna Sparks

Supervisor:

Prof. Emer. Margaretha D. Hofmeyr

Department of Biodiversity and Conservation Biology

University of the Western Cape

Co-supervisor:

Dr. Vanessa Couldridge

Department of Biodiversity and Conservation Biology

University of the Western Cape

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ABSTRACT

Homopus areolatus is an endemic terrestrial tortoise that resides in a Mediterranean type of climate, which is characterised by winter rainfall and mild winter temperatures. Within ectotherms, such as *H. areolatus*, physiological changes are elicited by changes in the ambient temperature. These physiological changes are evident in the blood profile of reptiles. I described the morphology of immature and mature erythrocytes, leukocytes as well as thrombocytes of *H. areolatus*. Additionally, I evaluated erythrocytes, leukocytes and thrombocytes to assess the effects of season and cohort on these cells. Blood samples were collected in 2000 and 2001 at Elandsberg Nature Reserve in the Western Cape from *H. areolatus* cohorts (female, male, juvenile) in all seasons (spring, summer, autumn, winter). Blood smears were made and stained with modified Giemsa stain. SigmaStat was used for all statistical analysis. Immature erythrocyte types within *H. areolatus* included basophilic rubricytes, polychromatophilic rubricytes and polychromatophilic erythrocytes. Upon my evaluation, I encountered evidence to suggest that small and large immature erythrocytes possibly developed from two distinctive lineages. Further research is required to discern which lineage gave rise to which immature erythrocyte type. Cohort had no effect upon immature erythrocytes. Erythropoiesis was most prevalent during winter and spring within *H. areolatus*. Aberrant features of erythrocytes appeared to be more prevalent during autumn, which signified the driest season with limited food and water. Mature erythrocytes play a huge role in oxygen transport and metabolism in individuals. Factors such as size and shape are relevant since small, mature, ellipsoidal erythrocytes transport oxygen more efficiently than large, spherical erythrocytes. In *H. areolatus* small, mature, ellipsoidal erythrocytes appeared to be most prevalent during spring and summer. During winter however, large, spherical erythrocytes appeared to be most prevalent. Thrombocytes and seven types of leukocytes were observed within *H. areolatus*, namely heterophils, lymphocytes, eosinophils, basophils, monocytes, plasma cells and azurophils. Among cohort and season heterophils were most prevalent overall, followed by lymphocytes and eosinophils respectively. Basophils, monocytes, plasma cells and azurophils were present but overall, were relatively few. *H. areolatus* appeared to be healthy, and leukocyte counts as well as its dimensions appeared to be in accordance with other reptilian studies. This study serves as the first baseline haematological reference for *H. areolatus*. The study forms the second of its kind on South African tortoises, only

one other haematological study has been done namely, *P. geometricus* which is a sympatric species to *H. areolatus*.

Date: 28 July 2015



DECLARATION

I declare that Blood cell histology of *Homopus areolatus*; effects of season and cohort is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

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Sharna Sparks

Date: 28 July 2015



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1 GENERAL INTRODUCTION

1.1 VERTEBRATE HAEMATOLOGY

A complete haematological evaluation involves measuring packed cell volume (PCV) and haemoglobin concentration (Hb), performing red blood cell and differential white blood cell counts, as well as doing a histological evaluation of blood cell types and prevalence (Campbell 2004). Of all the different haematological indices, a proper evaluation of a blood smear has the greatest value because such evaluations provide a wealth of diagnostic information (Stacy et al. 2011). The haematological values of animals are influenced by many intrinsic and extrinsic factors. Blood parameters often differ between sexes, e.g., male geometric tortoises have a higher PCV and larger erythrocytes than females have (Walton 2012). Parameters can also change with age; Stacy and Whitaker (2000) have shown that juvenile mugger crocodiles have lower erythrocyte counts and higher lymphocyte counts than adults have. Similarly, reproductive status can influence haematological values, for example, gestating *Egernia whitii* females have higher lymphocyte counts than postpartum females have (Cartledge et al. 2005). Seasonal fluctuations in blood values have been indicated in many species, e.g., values for erythrocytes, heterophils and lymphocytes of the yellow pond turtle are higher in summer than in the other seasons (Yu et al. 2013).

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It is widely recognised that haematological indices can be used as early indicators of pathology and disease, such as anaemia, inflammatory disease, parasite infestations, as well as haematopoietic and haemostatic disorders (Christopher et al. 1999; Campbell and Ellis 2007; Shadkhast et al. 2010; Stacy et al. 2011). Similarly, blood profiles (leukocyte levels) play a valuable role in showing environmentally induced physiological stress, which becomes more and more prevalent with habitat degradation of many species (Davis et al. 2008; Irwin et al. 2010; Johnstone et al. 2012). It is difficult, however, to distinguish disease conditions from normal physiological fluctuations when baseline reference values for haematological indices are not available (Jacobson 1994). The establishment of complete blood profiles is minimally invasive and of great value to assess the health and fitness of wild and captive animals (Parida et al. 2014). Unfortunately, reference values are available for few reptile species, particularly for chelonians in South Africa. Such studies should thus have a high priority, considering the high tortoise diversity in this country.

1.2 BLOOD COMPOSITION OF REPTILES

Blood is composed of plasma and cells. Plasma is the liquid part of the blood that contains water, electrolytes, glucose, proteins and several other substances in small quantities (Schneck 2003). Plasma is usually colourless in mammals, however, in reptiles, such as snakes, the plasma is green to yellow due to the high carotenoid and riboflavin content (Dessauer 1970). In mammals, red blood cells make up 40% of the blood volume (Khanna and Yadav 2005), whereas in reptiles, it can constitute between 20% and 40% of the blood volume (Dessauer 1970).

The blood of reptiles contains leukocytes, erythrocytes and thrombocytes. In contrast to mammalian red blood corpuscles and blood platelets, reptiles and other non-mammalian vertebrates have nucleated erythrocytes and thrombocytes (Shadkhast et al. 2010). Reptilian erythrocytes have a flattened, oval shape with a round, centrally placed nucleus (Armando and Rovira 2010). Reptilian erythrocytes contain haemoglobin tetramers and are involved in oxygen and carbon dioxide transport, as in other vertebrates (Strik et al. 2007). The life span of the reptilian erythrocyte is between 600 and 800 days (Nardini et al. 2013). This prolonged life span of erythrocytes is reportedly due to the slow metabolic rate of reptiles (Nardini et al. 2013). Reptiles have larger erythrocytes than birds and mammals have but they are smaller than in amphibians (Shadkhast et al. 2010).

Reptilian thrombocytes are relatively small cells and vary in shape from round to ellipsoid (Aughey and Frye 2001). The cytoplasm is colourless to pale blue and display irregular cytoplasmic margins when aggregated (Campbell 1996). The nucleus is usually centrally located and has a condensed chromatin network that stains purple (Campbell 1996). These cells can easily be mistaken for small lymphocytes and the darkly stained cytoplasm of lymphocytes can be used to distinguish the two cell types (Nardini et al. 2013). Reptilian thrombocytes have the same haemostatic role as the platelets do in mammals (Russel 2010).

Leukocytes are categorised as granulocytes and agranulocytes or mononuclear cells (Zhang et al. 2011; Nardini et al. 2013). In reptiles, the granulocytes include heterophils, eosinophils and basophils whereas the agranulocytes include lymphocytes and monocytes, with plasma cells and azurophils, respectively, being considered modified lymphocytes and monocytes (Nardini et al. 2013). Heterophils are relatively large, round cells, with transparent cytoplasm that contains eosinophilic

(pink to orange or reddish) spindle-shaped cytoplasmic granules (Arikan and Çiçek 2014). The nucleus of the heterophil is basophilic with dense chromatin (Javanbakht et al. 2013). The shape of the nucleus in reptiles varies from round to oval, and may be eccentrically located in crocodiles, snakes and chelonians (Strik et al. 2007). The nucleus of lizard heterophils may have two or more lobes (Strik et al. 2007). Heterophils are similar to neutrophils (in mammals) in both function and morphology (Javanbakht et al. 2013). Heterophils act as a defence mechanism against infections or inflammation and have the ability to phagocytize foreign material (Campbell 1996). Toxic heterophilia is associated with stress or disease conditions (Hawkey and Dennet 1989).

Reptilian eosinophils are large, round cells with round, eosinophilic cytoplasmic granules (Campbell 2004). The nucleus is central or eccentric with a round to elongated or bi-lobed shape (Campbell 2004). The function of eosinophils is to assist in the immune response in chelonians such as fighting off parasitic and bacterial infections, (Hawkey and Dennet 1989; Armando and Rovira 2010). Basophils are round cells that are relatively small, but are known to vary in size depending on the reptile species (Metin et al. 2008; Stacy et al. 2011). The cytoplasm of basophils is pale purple with large, round, dark-purple granules that obscure the nucleus (Stacy et al. 2011; Mayer and Donnelly 2013). The nucleus of the basophil is not lobed and may be centrally or eccentrically located (Zhang et al. 2011, Mayer and Donnelly 2013). In the immune system of individuals, basophils defend the body from chronic, long-term illnesses and associated inflammation (Hawkey and Dennet 1989). In addition, basophils help to heal and rid the body of haemoparasites such haemogregarines, trypanosomes and iridovirus infections (Strik et al. 2007).

Reptilian lymphocytes vary in size and typically contain large and small lymphocytes (Armando and Rovira 2010). Lymphocytes are round to oval in shape with scant basophilic cytoplasm and a high nucleus to cell ratio (Campbell 1996; Strik et al. 2007). The large nucleus is round and is either centrally or eccentrically located (Campbell 1996). The nucleus contains ring-like patterns of clumped chromatin that may be confused with nucleoli (Harvey 2012). Lymphocytes are important in the immune response and assist in wound healing and infectious or inflammatory diseases (Armando and Rovira 2010). Plasma cells are stimulated lymphocytes that are involved in severe infection or inflammatory disease (Nardini et al. 2013). The cells are large, round to oval in shape, and have a deeply basophilic cytoplasm with a

perinuclear halo (Nardini et al. 2013). The round to oval nucleus is eccentric, stains darkly blue, and has a condensed chromatin network (Frye 1991; Nardini et al. 2013).

Monocytes are the largest leukocytes in the peripheral blood of reptiles (Strik et al. 2007) and are round to oval in shape (Zhang et al. 2011). The cytoplasm is pale to light blue in colour and the nuclei are kidney-shaped, with a smooth to slightly clumped chromatin network (Strik et al. 2007). Reactive monocytes contain cytoplasmic vacuoles, which develop due to phagocytic activity in response to a systemic antigen (Stacy et al. 2011). Monocytes that leave the peripheral blood develop into macrophages in the tissues (Stacy et al. 2011) and are involved in granuloma and giant cell formation when the immune system attempts to wall off foreign substances (Strik et al. 2007). Monocytes of reptiles may contain fine azurophilic granules in the cytoplasm and these cells have been referred to as either azurophilic monocytes or azurophils. Some scientists regard azurophils as a distinct leukocyte type but most evidence point towards them being monocytes (Campbell and Ellis 2007). Azurophils are large, round cells with a round, oval or bi-lobed nucleus that is slightly eccentrically located. Azurophils are frequently found in snakes, squamates and crocodiles and only occasionally in chelonians (Strik et al. 2007).

1.3 BLOOD CELL DEVELOPMENT IN REPTILES

Haemopoiesis in reptiles is most prevalent in the bone marrow but has also been shown to occur in the liver, spleen and thymus (Campbell 2012; Arikan and Çiçek 2014). Haematopoietic stem cells give rise to two types of cell lineages namely the myeloid and lymphoid lineages (Sypek and Borysenko 1988; McGeady et al. 2006; Brody 2012). The myeloid progenitor is pluripotent and differentiates into more specialised progenitor cells, namely the thromboblats, rubriblasts, monoblasts and myeloblasts (Sypek and Borysenko 1988; Brody 2012). Thromboblats and rubriblasts are quite similar in appearance but the thromboblats are smaller than the rubriblasts. Both cell types have a large, round nucleus with nucleoli and scant, deeply basophilic cytoplasm (Campbell 2012). Through the process of thrombopoiesis, thromboblats produce immature thrombocytes that become progressively smaller and more oval with maturity (Campbell 2012). Rubriblasts go through several developmental stages (prorubricytes, basophilic rubricytes, early and late polychromatophilic rubricytes and polychromatophilic erythrocytes) during erythropoiesis before giving rise to mature erythrocytes (Campbell and Ellis 2007; Campbell 2012). During development, the shape of the cell changes from round to flattened ellipsoid, the cell becomes

progressively larger, and the chromatin becomes more condensed (Knotková et al. 2002; Campbell and Ellis 2007; Stacy et al. 2011; Campbell 2012). Monoblasts give rise to monocytes and macrophages through monocytopoiesis, but the developmental pathway is not clear in reptiles (Campbell 2012). The myeloblasts are large, round cells with basophilic cytoplasm and large nuclei that develop into granulocytes (Campbell 2012). With granulopoiesis, the cells decrease in size, the cytoplasm becomes less basophilic and specific cytoplasmic granules start to appear (Campbell 2012). The developmental stages include progranulocytes, myelocytes, metamyelocytes and band cells. Cell specific granules appear in the myelocyte stage and the final shape of the nucleus develops in band cells. The different granulocytes of reptiles include heterophils, eosinophils and basophils (Campbell 2012).

The lymphoid lineage of progenitor cells gives rise to lymphocytes. Blood-borne stem cells that lodge in the thymus produce the first lymphocytes in reptiles (Campbell 2012) but lymphopoiesis can also occur in the bone marrow, spleen and liver (Sypek and Borysenko 1988; Sano-Martins et al. 2002). Two precursor cell lineages respectively give rise to the B and T lymphocytes, which cannot be distinguished morphologically. The B lymphocytes differentiate further into plasma cells (Theml et al. 2004; Campbell 2012). Immature lymphocytes, the lymphoblasts and prolymphocytes, are larger than mature lymphocytes. These immature cells have large nuclei and the cytoplasm is deeply basophilic (Campbell and Ellis 2007; Campbell 2012).

There are reports that erythrocytes in reptiles may develop from a second origin, distinct from the rubriblast lineage, giving rise to two lineages with different morphological characteristics such as size differences (Pienaar 1962; Frye 1991). Pienaar (1962) proposed that mature erythrocytes stem from a lymphoid lineage apart from the erythrocyte stem cell lineage, whereas Frye (1991) identified thrombocytes as a second source of erythrocyte production. After an intensive search of the literature, I could not find support for the findings of either of these two authors; in fact, it appears if subsequent researchers ignored these reports. Consequently, an explanation for the observations of Pienaar (1962) and Frye (1991) may lay elsewhere.

The nomenclature of immature erythrocytes in the circulating blood of reptiles is contentious. Pienaar (1962) attempted to standardise names for blood cells and their developmental stages and classified erythrocytes into six developmental stages (Table 1.1). Frye (1991) used only five stages, which show some correspondence to

the classification of [Pienaar \(1962\)](#) but he did not include the basophilic normoblast stage. In recent years, the terminology for vertebrate erythrocytes has again been standardised, as is presented by [Campbell \(2012\)](#). My descriptions will adhere to the recent nomenclature ([Table 1.1](#)), as in [Campbell \(2012\)](#), but I did not see all developmental stages in peripheral blood.

Table 1.1 Nomenclature of erythrocyte developmental stages.

Pienaar (1962)	Frye (1991)	Campbell (2012)
Pro-erythroblast	Pro-erythroblast	Rubriblast
Erythroblast	Erythroblast	Prorubricyte
Basophilic normoblast		Basophilic rubricyte
Polychromatophilic normoblast	Early polychromatophil	Early polychromatophilic rubricyte
	Late polychromatophil	Late polychromatophilic rubricyte
Pro-erythrocyte		Polychromatic erythrocyte
Mature erythrocyte	Mature erythrocyte	Mature erythrocyte

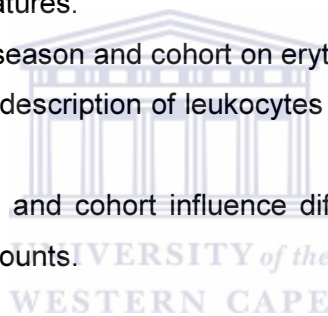
1.4 STUDY ANIMAL AND STUDY OBJECTIVES

South Africa has the richest tortoise diversity in the world with 13 species and five genera. The five genera are *Chersina*, *Stigmochelys*, *Kinixys*, *Psammobates* and *Homopus* ([Hofmeyr et al. 2014](#)). *Homopus areolatus*, commonly known as the parrot-beaked tortoise, is endemic to South Africa and is found all along the south-western and southern regions of the country where it is exposed typically to a Mediterranean climate ([Alexander and Marais 2007](#)). Although most of its range falls within the Fynbos Biome, *H. areolatus* also penetrates the Albany Thicket Biome in the easternmost parts of its range ([Hofmeyr et al. 2014](#)).

Factors such as habitat destruction, degradation and fragmentation, climate change, alien invasion, and subsidised predators can affect the health of tortoises and cause the decline of populations ([Davis et al. 2008](#); [Perpiñán et al. 2008](#); [Barrows 2011](#); [Zhang et al. 2011](#)). The responses of reptiles to environmental alterations can be manifested as physiological stress, which is reflected by changes in certain blood parameters ([Paul et al. 2008](#)). Because cohort and seasonal fluctuations in environment conditions can also alter blood parameters ([Deem et al. 2009](#)), it is

necessary to establish baseline haematological values to distinguish normal fluctuations from disease or physiological stress (Deem et al. 2009). In order to establish reference values, one needs to study the effects of season and cohort on blood parameters and simultaneously explore normal levels of haemoparasite infestation. Baseline haematological values exist for only one South African tortoise species, *Psammobates geometricus*. The goal of the present study was to develop baseline haematological information for a second endemic tortoise in South Africa, namely *H. areolatus*. Such information could be used to assess the health status of other populations in the wild, particularly in degraded habitats, or of individuals in captivity. To obtain this goal, I collected blood over four seasons from all *H. areolatus* cohorts for a complete histological evaluation and set the following objectives:

- 1) Provide a histological description of erythrocyte developmental stages and aberrant features in peripheral blood.
- 2) Assess the effects of season and cohort upon the prevalence of erythrocyte types and aberrant features.
- 3) Assess the effects of season and cohort on erythrocyte size and shape.
- 4) Provide a histological description of leukocytes and thrombocytes in peripheral blood.
- 5) Evaluate how season and cohort influence different leukocyte types through differential white cell counts.



2 DESCRIPTION AND PREVALENCE OF ERYTHROCYTE TYPES

2.1 INTRODUCTION

The erythrocytes of fish, amphians, reptiles and birds have nuclei, unlike the red blood corpuscles of mammals (Strik et al. 2007). Similar to other vertebrates, the erythrocytes of reptiles contain haemoglobin that is responsible for oxygen and carbon dioxide transport to and from the cells in the body (Strik et al. 2007). Erythrocytes are thus central to the physiology of animals, and a clear understanding of the cells' morphology, aberrations as well as the intrinsic and extrinsic factors that affect these cells are crucial in distinguishing between natural physiological processes and disease conditions (Campbell 2004; Zhang et al. 2011; Weiser 2012; Javanbakht et al. 2013).

The erythrocyte developmental pathways of all vertebrates are relatively similar despite the fact that mammalian erythrocytes lose their nuclei during the final stages of development (Kingsley et al. 2004; Campbell and Ellis 2007). All blood-borne cells originate from pluripotent haematopoietic stem cells, which give rise to lymphoid and myeloid progenitor cells with more limited developmental potential (Nabity and Ramaiah 2012). The lymphoid progenitor cells develop into lymphocytes and plasma cells (Theml et al. 2004). The myeloid progenitor cells include the myeloblasts, monoblasts, thromboblats and rubriblasts, which respectively give rise to granulocytic leukocytes, monocytes, thrombocytes and erythrocytes (Chow and Frenette 2014).

Erythropoiesis in adult reptiles occurs mainly in bone marrow and in recent years, most researchers recognise seven erythropoietic developmental stages: the rubriblasts, prorubricytes, basophilic rubricytes, early and late polychromatophilic rubricytes, polychromatophilic erythrocytes and mature erythrocytes (Campbell 2012). The overall developmental trends are for the cells to become progressively larger with more cytoplasm that changes from deeply basophilic to eosinophilic as haemoglobin synthesis progresses, and for the cell shape to change from round to flattened ellipsoidal (Campbell and Ellis 2007; Campbell 2012). Most reptiles release immature erythrocytes from the rubricyte stage onwards in their blood circulation (Campbell and Ellis 2007; Campbell 2012), but polychromasia (the occurrence of immature erythrocytes in the circulation) is particularly prevalent in young animals and individuals with regenerative anaemia (Stacy et al. 2011; Campbell and Ellis 2007).

Apart from developing from myeloid progenitor cells, two independent reports indicate that reptilian erythrocytes can have another origin; i.e., either from a lymphoid lineage (Pienaar 1962) or from a thrombocyte lineage (Frye 1991). Pienaar (1962) proposed that erythrocytes are primarily derived from lymphocytes and secondarily from myeloid stem cells. According to his view, lymphocytes from the blood are filtered out in the bone marrow where a large proportion of these cells are transformed into a lymphocyte-erythrocyte series. Some of the immature cells of the lymphoid lineage may escape into the peripheral blood and the early stages of development are characteristically smaller than immature erythrocytes from the myeloid stem cell lineage. According to Pienaar (1962), myeloid erythropoiesis only becomes significant under conditions of stress when an increase in the number of erythrocytes is required. Pienaar (1962) did his study at a time when it was generally believed that lymphocytes are circulatory stem cells (haemocytoblasts) that can differentiate into other blood cells in the spleen and bone marrow. Although this view is no longer held, Pienaar (1962) was the first to suggest that erythrocytes may be derived from more than one lineage.

Subsequent to Pienaar's study, Frye (1991) reported the existence of two erythrocyte lineages in reptiles and proposed that in addition to rubriblasts, thrombocytes can also transform into erythrocytes. He concluded that thrombocytes are pluripotent after benzidine peroxidase stains indicated the presence of haemoglobin in thrombocytes that are transforming into erythrocytes. The need for this transformation emerges during conditions of acute and chronic blood loss (Aughey and Frye 2001). A second line of erythrocyte formation, from lymphocytes and/or thrombocytes, may explain reports that immature and mature erythrocytes in peripheral blood of reptiles can vary greatly in size (Pienaar 1962; Vasse and Beaupain 1981; Frye 1991; Jacobson 2007; Walton 2012; Fraser 2013).

Reptilian erythrocytes have a long lifespan of 600 to 800 days (Campbell 1996; Nardini et al. 2013), probably due to the slow metabolic rate of reptiles (Dessauer 1970). Senescent cells in the peripheral blood are often larger than average mature erythrocytes (Nusse et al. 2013). When erythrocytes become senescent, the cytoplasm starts to swell, whereafter the nucleus first becomes perfectly round before it turns pyknotic. The cytoplasm of ageing erythrocytes may lose its colour and becomes transparent, whereafter they are called ghost cells (Nardini et al. 2013).

Histological evaluations of reptile blood do not only provide information on the prevalence of different developmental stages in the blood smear, but also on the occurrences of aberrant morphological features of erythrocytes. In many instances, morphological aberrations of erythrocytes have no pathological significance, but they may be indicative of inflammation, toxicosis, parasite infestations or regenerative anaemias (Nardini et al. 2013). Polychromasia may not necessarily indicate an aberration, e.g., young individuals have a higher level of polychromasia than adults do (Stacy et al. 2011), but it can be indicative of disease, for example, high levels of polychromasia could indicate an irregular regenerative response (Martinho 2012). Because immature erythrocytes are capable of replication, high levels of polychromasia are often associated with the occurrences of binucleated erythrocytes and mitotic figures in erythrocytes of the peripheral blood (Nardini et al. 2013).

Aberrant features relating to cell size and shape include poikilocytosis, anisocytosis, macrocytosis and microcytosis (Tkachuk et al. 2002). Poikilocytosis refers to variation in the shape of erythrocytes whereas anisocytosis denotes variation in erythrocyte size (Tkachuk et al. 2002). Macrocytosis refers to the presence of abnormally large cells and microcytosis indicates the presence of abnormally small erythrocytes (Tkachuk et al. 2002). Erythrocytes that do not contain nuclei, the erythroplastids, are not common in reptiles and their presence do not appear to have a clinical significance in healthy animals (Campbell 2004; Stacy et al. 2011). Reptilian erythrocytes often have small, basophilic inclusions in the cytoplasm, which are usually explained as staining artefacts, or degenerating organelles, which may be linked to erythrocyte senescence (Campbell 2004; Pendl 2006; Stacy et al. 2011; Campbell 2012). These basophilic inclusions, however, may also reflect a manifestation of the regenerative response to anaemia (Nardini et al. 2013). Cytoplasmic vacuoles are likewise observed in the cytoplasm of erythrocytes and are usually considered drying artefacts, although they may be associated with haemoparasite infestations (Campbell 2004; Pendl 2006).

Several studies have shown that environmental factors and cohort can influence the prevalence of polychromasia and aberrant erythrocyte features in reptiles (Pienaar 1962; Duguay 1970; Campbell 2004; Pendl 2006; Stacy et al. 2011). In many reptile species, juveniles have more immature erythrocytes in the circulation than adults have (Campbell 2012), or juveniles and males have a higher degree of polychromasia than in females (Walton et al. 2013). Increased levels of erythropoiesis with a concomitant increase in the occurrence of immature erythrocytes in the peripheral circulation have

been reported for hibernating and post-hibernating reptiles (Pendl 2006; Campbell 2012). Pienaar (1962) noted that South African reptiles show a high degree of erythropoiesis during winter and linked this phenomenon to hibernation. However, not all reptiles in South Africa hibernate in winter and Walton et al. (2013) ascribed the elevated winter levels of immature erythrocytes in *P. geometricus* to a physiological response to increased food availability and preparation for increased activity levels when temperature increases in spring.

Few haematological studies have been done on chelonians (Arkan and Çiçek 2010) and little is known of the blood cell parameters of South African tortoises. Pienaar (1962) included only one specimen for each of *H. areolatus* and *Stigmochelys (Geochelone) pardalis* in his study on the haematology of South African reptiles. Consequently, he did not evaluate the effects of cohort or season on the blood cell parameters of these species. To date, the haematology of only one South African tortoise species, *P. geometricus*, has been studied in detail (Walton 2012). South Africa has a high tortoise diversity, which is of great conservation importance. More studies are thus essential to establish baseline haematological values for South African tortoises. Such information will help scientists and conservationists to distinguish healthy and normal haematological reference values from those that indicate disease or stress.

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Homopus areolatus is a small-bodied tortoise, endemic to South Africa, that prefers a Mediterranean-type climate and lives in close association with Fynbos vegetation in the southwestern and southern coastal regions of the Western and Eastern Cape Provinces (Branch 1989; Boycott and Bourquin 2000; Alexander and Marais 2007). Much of this species' habitat is threatened by urban and agricultural development (Rouget et al. 2004); consequently, populations in some regions are relegated to sub-optimal habitat (M.D. Hofmeyr, personal communication). Because environmental stress may impact the health of populations (Christopher et al. 1999; Barrows 2011; Zhang et al. 2011), it is important to establish baseline haematological values that can be used in conservation assessments. To this end, I studied the haematology of a wild *H. areolatus* population, living in optimal habitat at Elandsberg Nature Reserve, in order to establish reference haematological values for this species. My research objectives for this chapter were to (1) describe the histological features of erythrocyte developmental stages of *H. areolatus* in peripheral blood, (2) evaluate the effects of cohort and season on erythrocyte development and aberrations, and (3) compare values for *H. areolatus* with those of a close relative, *P. geometricus*, and distant

lineages from other regions in the world. Such comparisons should elucidate similarities or differences among chelonian lineages.

2.2 MATERIALS AND METHODS

2.2.1 Field procedures

Blood samples for *H. areolatus* were collected over four seasons from 2000 to 2001 at Elandsberg Nature Reserve (3 800 ha; 33° 26' S; 19° 01' E) in the south-western Cape, South Africa. In total, 84 samples were collected for male, female and juvenile tortoises (Table 2.1). Weather data for the corresponding period were obtained from AgroMet-ISCW for the nearest weather station, De Hoek (33.15° S, 19.03° E), 25 km north of Elandsberg, which has a similar orientation to the Elandsberg mountain range as the study site.

Table 2.1 Field trip dates and sample sizes of *Homopus areolatus* females, males and juveniles at Elandsberg Nature Reserve, South Africa.

Season	Dates (2000-2001)	Female	Male	Juvenile	Total
Spring	30 August - 16 September	11	5	5	21
Summer	11-19 December	10	8	2	20
Autumn	2-10 April	8	10	4	22
Winter	19-27 June	11	5	3	19

Tortoises were captured by hand, weighed to the nearest 0.1 g with an Ohaus digital balance (O'Haus Corporation, Florham Park, New Jersey, USA), whereafter blood was collected immediately to prevent stress-induced changes in blood parameters. The maximum volume of blood taken was determined by the weight of the animal and did not exceed 0.5% of the animal's field body weight, in line with conservative veterinary standards (Strik et al. 2007). A 25 G needle with a 1 or 2 ml syringe was used to collect blood from either the jugular vein or carotid artery. Sampling time was approximately 2 minutes and sampling attempts were terminated when not successful within 5 minutes. Heparin rather than EDTA was used as an anticoagulant because it is known that EDTA causes lysis of chelonian cells (Harding et al. 2005; Knotek 2006). Heparin, on the other hand, is known to impart a blue tinge to blood smears and to affect cell clumping (Houwen 2000; Strik et al. 2007).

Blood collected were kept on ice and blood smears were made within 1-12 hours of sampling. Blood smears were prepared in duplicate by the wedge-smear technique

with a glass slide spreader used only once (Houwen 2000). The smears were air-dried, fixed in absolute methanol for 10 minutes and subsequently stored in dust-free boxes. The animals were kept under observation for 24 hours, with access to drinking water during the dry season, before being returned to their capture site. The scutes of all individual tortoises were filed with a unique number (Honegger 1979) before release in order to ascertain if the same individual was recaptured in successive seasons.

2.2.2 Staining blood smears

Houwen (2000) recommended that blood films be fixed and stained immediately after the films are dry to obtain optimal results. In this study, the methanol-preserved blood smears were stored for several years before being stained, and initial staining results were poor. The staining ability of the smears improved drastically, however, after I dipped the smears into methanol for 10 seconds before starting the staining procedure. I experimented with various Romanowsky stain recipes containing Wrights, May-Grünwald and Giemsa stains and found that the 48900 Giemsa Fluka modified stain solution yielded the best results. The Giemsa Fluka modified stain solution (Sigma-Aldrich, St. Louis, USA) consists of azure B, azure II-eosin, and methylene blue in a 1:12:2 weight ratio. Although the staining instructions for Giemsa Fluka was to use a 1:20 dilution, I found that 1:10 dilution worked best. I also experimented with different pH buffers and staining durations before selecting buffer tablets ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0,47g/l and KH_2PO_4 , 0.47g/l; Merck Pty Ltd, New Jersey, USA) with pH 6.8 and a staining time of 60 minutes.

Fresh stain solutions were made up each week. I first filtered the Giemsa Fluka stain and diluted it in a 1:10 ratio. I used coplin jars for multi-slide staining and submerged the slides for 60 minutes. After staining, the slides were given a quick rinse with distilled water and left to air-dry. Once the slides were completely air-dried, I inspected the slides for staining intensity and then cover-slipped them using Entellan new rapid-mounting medium (Merck Pty Ltd, New Jersey, USA).

2.2.3 Histological evaluation of erythrocyte development

When doing the histological evaluation of erythrocytes, I used a Leica DM 500 digital photomicroscope (SMM Instruments, Pty, Ltd) with a 10x eyepiece and a 40x objective to give 400x magnification, or a 100x objective to give 1000x magnification under immersion oil. The microscope was linked with Leica LAS software version.1.8.0 (Leica Microsystems Ltd., Heerbrugg, Switzerland) to a Leica ICC50

camera (Wetzlar, Germany) to photograph developmental stages. I considered a number of characteristics to classify each erythrocyte as immature, mature or senescent. I looked at the size and shape of the cell, the nuclear to cellular ratio, and the staining intensity of the cytoplasm and nucleus. I also examined the chromatin network in the nucleus to assess whether it was condensed or dispersed. I did not detect the first two developmental stages of erythrocytes (rubriblasts and prorubricytes) in the blood smears and could not distinguish clearly between early and late polychromatophilic rubricytes. Consequently, I classified immature erythrocytes into three categories according to their level of maturity, namely basophilic rubricytes, polychromatophilic rubricytes and polychromatophilic erythrocytes.

Using the meandering technique, I explored the blood smears of each *H. areolatus* individual (three cohorts) collected over four seasons. The meandering technique was done with the 40x objective lens and I inspected each slide from left to right and back again to cover the whole smear. Whenever I encountered a cell that showed the typical characteristics of a particular erythrocyte type, I switched to the 100x objective to study the cell histology in detail for descriptive purposes and to photograph the cell for a permanent record. I also took photographs at 100x magnification to allow measurements of cell parameters for 30 cells of each developmental stage. All images were saved as jpeg files (2048 x 1536 pixels).

I measured and analysed the photographs of erythrocyte developmental stages with Nikon NIS Elements imaging software (Basic Research version 3.10 Inc., Nikon Instruments, Europe B.V., AS Amstelveen, The Netherlands). The parameters that were measured automatically for both the cell and nucleus included area (surface area in μm^2), length (the longest axis in μm), width (calculated from area/length in μm), elongation (determined from a set of Feret's diameters between 0 and 180 degrees with 10 degree angle intervals as MaxFeret/MinFeret), and mean intensity (the statistical mean of intensity values of pixels). These parameter definitions are in accordance with the glossary of Nikon NIS Elements.

Before taking measurements, pixel size was manually calibrated to a micrometer scale so that 1 pixel corresponded to 0.064 μm for images captured at 1000x magnification. Each image was then optimised to reduce staining artifacts and to improve cellular and nuclear boundaries. The Nikon NIS Elements system uses pixelation to distinguish between light and dark images, therefore I used the contrast function to distinguish the nucleus from the cytoplasm. After contrast adjustments, the

nucleus was darker than the cytoplasm. I adjusted the white saturation intensity to wash out staining artifacts that might be present in the background on the slide. The “auto detect” threshold function (from the binary toolbar of NIS system) was then used to obtain the best measurement to further resolve nuclear and cellular boundaries. Subsequently, I used the “erode” or “open” function of NIS to select a cell and match cellular and nuclear boundaries with threshold values. NIS Elements digitally computerized measurements to an accuracy of 0.01 μm and exported measurements to a Microsoft Excel 2007 spreadsheet for further analyses.

2.2.4 Quantifying erythrocyte types and features

I evaluated the prevalence of erythrocyte types and features while doing differential white cell counts on all individuals using the meandering technique. For this procedure I also used a Leica DM 500 photomicroscope at 1000x magnification under immersion oil. I counted and identified 100 white cells and whilst doing that took cognisance of the abundance of specific erythrocyte types and features. After finishing the white cell count, I ranked the prevalence of cell types and features from zero to three with zero representing absence, one representing a low abundance, two representing an intermediate presence, and three representing a high abundance. The cell types noted included rubricytes, polychromatophilic erythrocytes, senescent erythrocytes, macrocytes, microcytes and erythroplastids. The features noted included the degree of poikilocytosis, and the presence of intracellular vacuoles, cytoplasmic inclusions as well as intracellular parasites.

2.2.5 Data and statistical analysis

All developmental stages ($N = 30$ per type) were combined in order to test if there were differences among stages for the measured parameters. I used a one-way ANOVA (F statistic) for parametric data and a Kruskal-Wallis ANOVA on ranks (H statistic) for non-parametric data. Both tests were followed by Student-Newman-Keuls post hoc comparisons. The data for erythrocyte types and features were assessed for the effects of cohort and season but two-way ANOVAs were not possible because the data were not parametric. Consequently, I used one-way ANOVAs to evaluate the effects of season and cohort separately. Parametric one-way ANOVAs were followed by Student-Newman-Keuls post hoc comparisons whereas Dunn’s post hoc comparisons were the best option for ranked data that did not pass the requirements for parametric ANOVAs. I considered a difference as significant at $P \leq 0.05$ but adjusted the P -value, using the sequential Bonferroni procedure, where multiple tests

were done (Quinn and Keough 2002). SigmaStat (SPSS Inc., Chicago, U.S.A. version 2.03) was used for all statistical evaluations.

2.3 RESULTS

2.3.1 Weather conditions

The study site has a Mediterranean climate with winter rainfall and mild winter temperatures. In both 2000 and 2001, rainfall was abundant between May and September (Fig. 2.1a). Maximum and minimum temperatures for 2000 and 2001 followed approximately the same pattern with high temperatures in December to March and low temperatures between May and September (Fig. 2.1b).

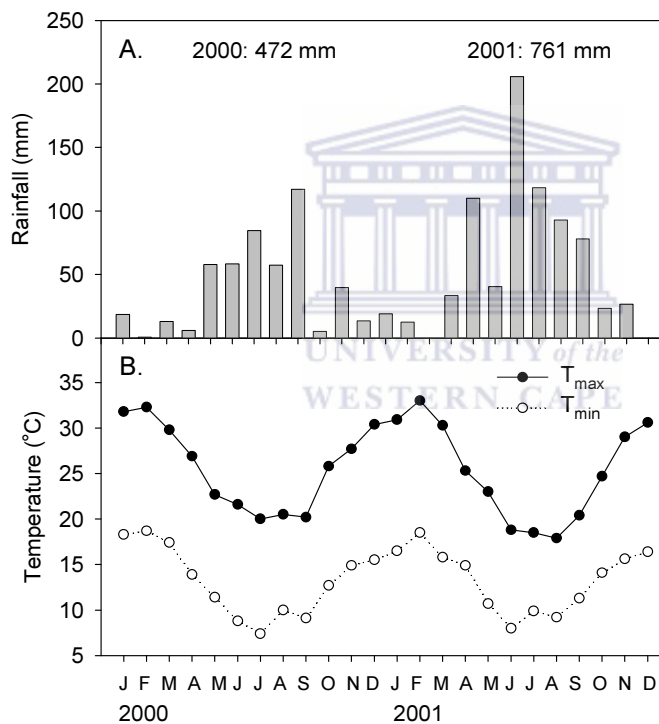


Figure 2.1 (a) Monthly rainfall (mm), with annual rainfall for each year, and (b) maximum and minimum temperatures in 2000 and 2001 for De Hoek weather station, 25 km from Elandsberg Nature Reserve.

2.3.2 Description of erythrocyte developmental stages

Erythrocyte developmental stages identified in the peripheral blood of *H. areolatus* included immature, mature and senescent (senile) cells. I distinguished three immature stages, namely basophilic rubricytes, polychromatophilic rubricytes and polychromatophilic erythrocytes in order of development. I did not detect the first two

developmental stages (rubriblasts and prorubricytes) and could not distinguish clearly between early and late polychromatic rubricytes because the development of immature stages is a continuous process. I relied mainly on size, shape, staining qualities, and nuclear to cellular ratios to distinguish the three categories.

Basophilic rubricytes were small spherical cells, each with a large, round nucleus (Fig. 2.2 a), giving it a high nucleus to cellular ratio. The cytoplasm was homogeneously basophilic while the nucleus stained a darker, more intense blue than the cytoplasm. The chromatin network stained dark-blue and were partially clumped, while the parachromatin stained a lighter blue colour (Fig. 2.2 a,b). Although most basophilic rubricytes were small cells, I occasionally detected substantially larger cells with characteristics that correspond to that of basophilic rubricytes (Fig. 2.2 b, c).

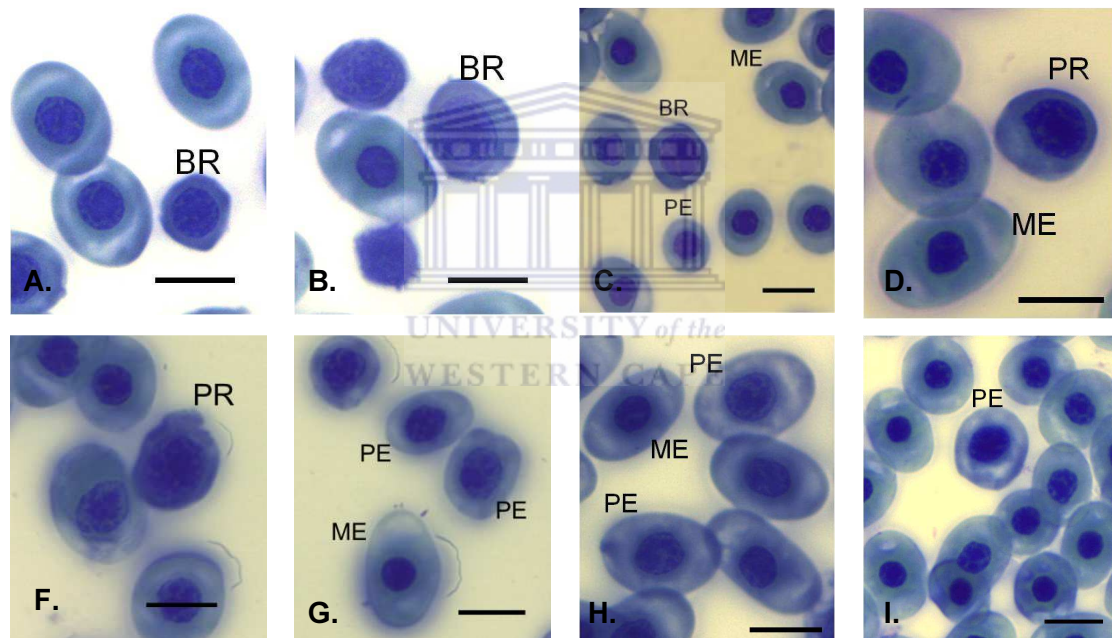


Figure 2.2 Immature developmental stages of erythrocytes in *Homopus areolatus*. BR represents basophilic rubricytes, PR represents polychromatophilic rubricytes, PE represents polychromatophilic erythrocytes and ME represents mature erythrocytes. The scale represents 10 μm at 1000x magnification.

Polychromatophilic rubricytes were larger than the basophilic rubricytes, but resembled the basophilic rubricytes in both cell and nuclear shape. The nuclear to cellular area ratio was noticeably lower than it was in the basophilic rubricytes (Fig. 2.2 d). The nucleus stained dark blue and the chromatin network appeared moderately condensed and clumped. The cytoplasm appeared mottled, with

basophilic and acidophilic sections, compared to the basophilic rubricytes (Campbell 2012; Fig. 2.2 d). Similar to the basophilic rubricytes, I occasionally detected polychromatophilic rubricytes that were substantially larger in size (Fig. 2.2 e).

The polychromatophilic erythrocytes were relatively large cells and the shape was distinctly more oval (Fig. 2.2 f). As was the case with basophilic rubricytes and polychromatophilic rubricytes, there was occasional variation in size (Fig. 2.2 f, g). The nucleus relative to the cell area continued to decrease. The chromatin network was more condensed in comparison to the basophilic and polychromatophilic rubricytes (Fig. 2.2 h). The staining intensity of the cytoplasm was less intense with a larger proportion of lighter blue than in the basophilic and polychromatophilic rubricytes (Fig. 2.2 f-h). The mottled appearance of the cytoplasm helped to identify these cells as immature.

There were clear differences between the mature and immature erythrocyte stages of *H. areolatus*. The mature cells were larger than the immature erythrocytes and the shape of the mature erythrocyte was more ellipsoid than the immature cells (Fig. 2.3 a). The nucleus of mature cells was small, giving a low nucleus to cell area ratio. The nucleus appeared pyknotic and the chromatin network was fully condensed (Fig. 2.3 a). The staining intensity of the cytoplasm was less intense and appeared a more homogeneously light blue (Fig. 2.3 b).

When mature cells reached the end of their life cycle they become old and senile. Senescent cells were larger than both the mature and immature erythrocytes (Fig. 2.3 c, d). The shape of the senile cells varied from round to oval depending on the stage of senescence (Fig. 2.3 c-e). The nuclei of the senile cells stained a lilac colour and depending on the senile phase, could be large or small. The cytoplasm of senescent cells did not absorb much stain and often appeared light green (Fig. 2.3 c-e). As the senile cell reached the end of its cycle, the cell often swelled and increased in size (Fig. 2.3 f). The cytoplasm of the cell became transparent and when only the nucleus remains visible, the cell is known as a ghost cell (Fig. 2.3 g-h).

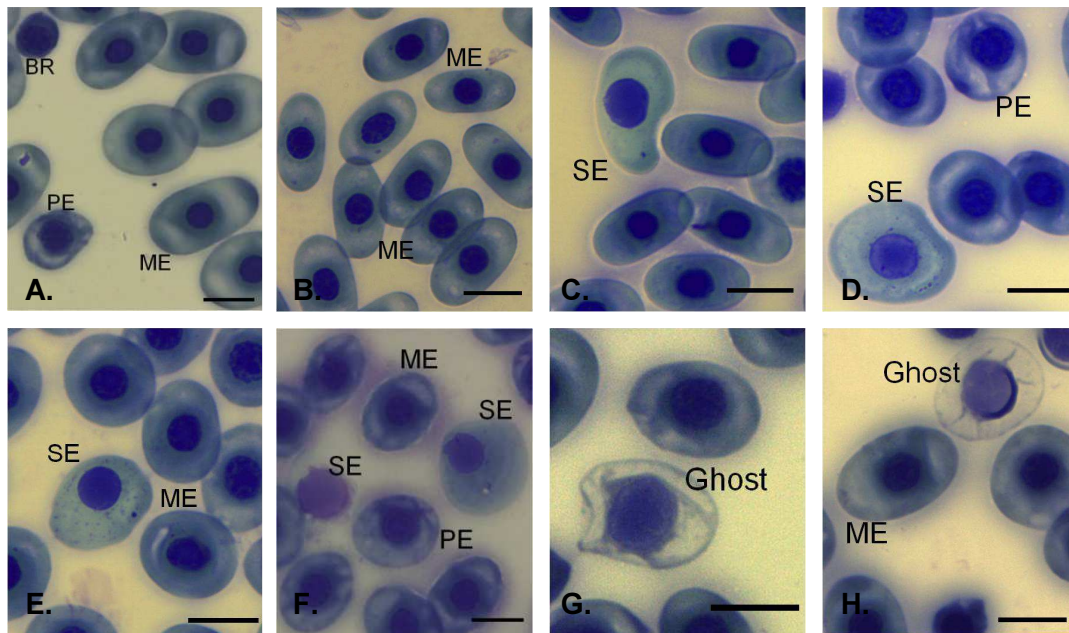


Figure 2.3 Mature and senile erythrocytes of *Homopus areolatus* with ghost cells. BR represents basophilic rubricytes, PE represents polychromatophilic erythrocytes, ME represents mature erythrocytes and SE represents senile erythrocytes. The scale represents 10 μm at 1000x magnification.

2.3.3 Size, shape and staining differences of developmental stages

The erythrocyte developmental stages of *H. areolatus* differed in size and shape. Cell area declined in size from senescent erythrocytes, through mature erythrocytes, polychromatophilic erythrocytes, polychromatophilic rubricytes and basophilic rubricytes ($F_{4,145} = 49.83$, $P < 0.0001$; Fig. 2.4). In contrast, nuclear area did not differ among all developmental stages. Nuclear area was largest and did not differ for senile erythrocytes, basophilic rubricytes and polychromatophilic rubricytes but they all had larger nuclear areas than polychromatophilic erythrocytes, which in turn had larger nuclei than mature erythrocytes ($H_4 = 74.00$, $P < 0.0001$; Fig. 2.4). Due to changes in cellular and nuclear areas of developmental stages, the nuclear to cellular ratios differed among all stages. The ratio was the largest for basophilic rubricytes (median, 25%, 75%: 0.43, 0.39, 0.48), followed by polychromatophilic rubricytes (0.35, 0.32, 0.38), polychromatophilic erythrocytes (0.27, 0.23, 0.30), senile erythrocytes (0.22, 0.19, 0.24) and mature erythrocyte (0.15, 0.13, 0.17). These changes in nuclear to cellular ratios are clearly demonstrated in the photographs of developmental stages (Figs. 2.2 and 2.3).

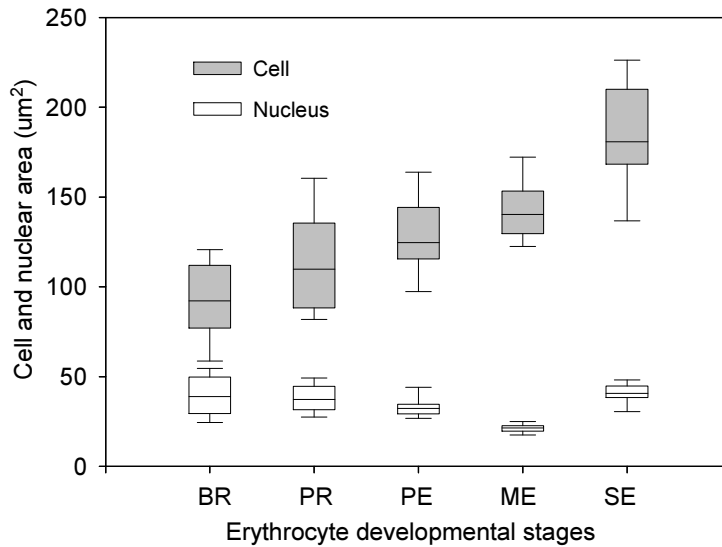


Figure 2.4 Cell and nuclear areas of *Homopus areolatus*. For the five erythrocyte developmental stages, BR represents basophilic rubricytes, PR represents polychromatophilic rubricytes, PE represents polychromatophilic erythrocytes, ME represents mature erythrocytes and SE represents senile erythrocytes. The box plot indicates the median, 25% and 75% percentiles, and the whiskers represent 10% and 90% percentiles. Data for cell areas were parametric but are indicated as medians to allow direct comparison with nuclear areas.

Both cell length and width differed among developmental stages. Cell length was highest and similar for mature and senescent erythrocytes, which were longer than all other stages, with no difference between polychromatophilic erythrocytes and polychromatophilic rubricytes, which were in turn longer than basophilic rubricytes ($F_{4,145} = 57.04$, $P < 0.0001$; Fig. 2.5a). Width of senescent erythrocytes was greater than, and basophilic rubricytes were narrower than, all other developmental stages. The width of polychromatophilic erythrocytes and polychromatophilic rubricytes did not differ, but polychromatophilic erythrocytes were wider than mature erythrocytes whereas there was no difference in width between polychromatophilic rubricytes and mature erythrocytes ($F_{4,145} = 28.80$, $P < 0.0001$; Fig. 2.5a). Nuclear length and width also differed among developmental stages. Nuclear length was lowest for mature erythrocytes and did not differ among the other stages ($H_4 = 69.85$, $P < 0.0001$). Nuclear width was also lowest for mature erythrocytes. In addition, nuclear width for senescent erythrocytes was higher than for all other stages, whereas width for

basophilic rubricytes and polychromatophilic rubricytes did not differ but was greater than that of polychromatophilic erythrocytes ($H_4 = 73.14$, $P < 0.0001$; Fig. 2.5b).

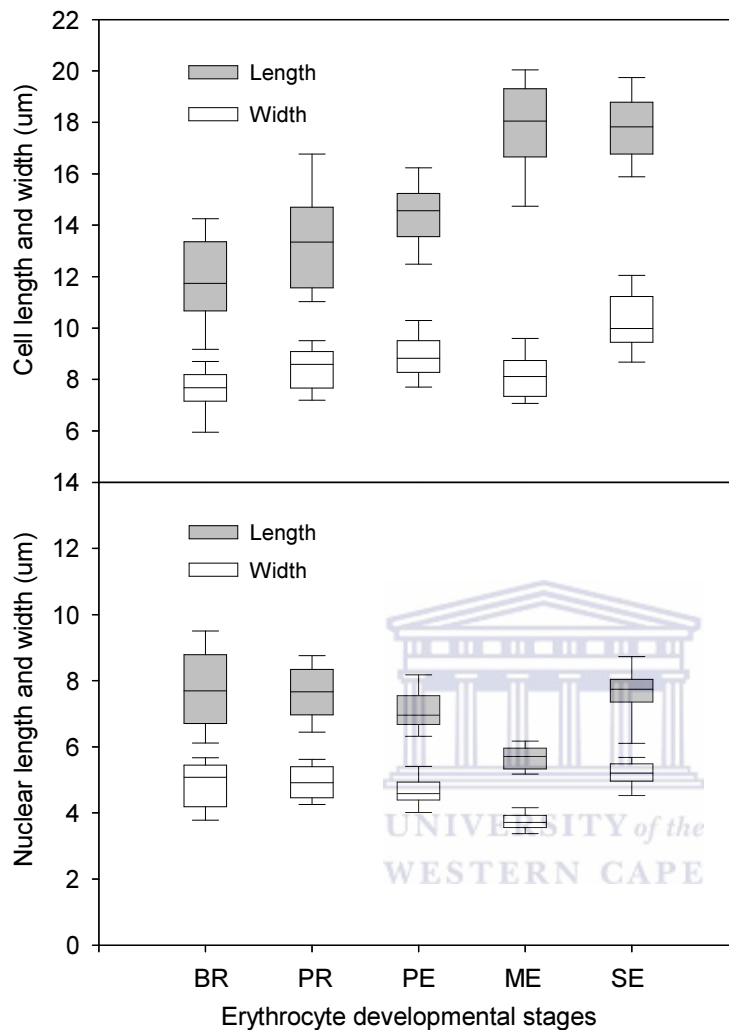


Figure 2.5 (a) Cell and nuclear length and width of *Homopus areolatus* for the five developmental stages (BR represents basophilic rubricytes, PR represents polychromatophilic rubricytes, PE represents polychromatophilic erythrocytes, ME represents mature erythrocytes and SE represents senile erythrocytes). The box plot indicates the median, 25% and 75% percentiles and the whiskers represent 10% and 90% percentiles.

Shape differences among erythrocyte developmental stages were evident from differences in cell elongation ($H_4 = 83.10$, $P < 0.0001$; Fig. 2.6). Mature erythrocytes were most elongated and basophilic rubricytes least elongated. Senescent erythrocytes had the second largest elongation followed by polychromatophilic

erythrocytes and polychromatophilic rubricytes, which did not differ. Shape, as elongation, differed only slightly for the nuclei, with the nuclei of all developmental stages having a similar shape except for that of senescent erythrocytes, which were least elongated ($H_4 = 13.55$, $P = 0.0089$; Fig. 2.6).

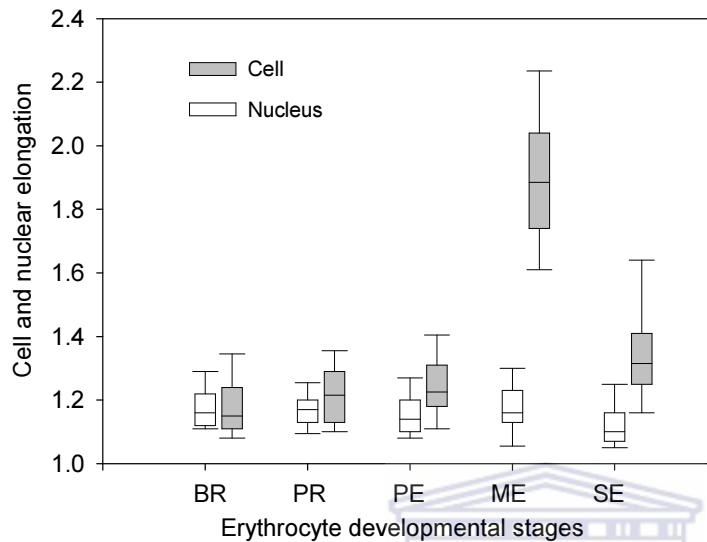


Figure 2.6 Cell and nuclear elongation of *Homopus areolatus* for the five developmental stages (BR represents basophilic rubricytes, PR represents polychromatophilic rubricytes, PE represents polychromatophilic erythrocytes, ME represents mature erythrocytes and SE represents senile erythrocytes). The box plot indicates the median, 25% and 75% percentiles and the whiskers represent 10% and 90% percentiles.

Staining differences among developmental stages were evident as pixelation (mean intensity) differences for both the cell ($F_{4,145} = 182.34$, $P < 0.0001$) and nucleus ($F_{4,145} = 26.57$, $P < 0.0001$; Fig. 2.7). Pixelation was highest for senescent cells (meaning that they stained least), followed by equal pixelation for mature and polychromatophilic erythrocytes, then polychromatophilic rubricytes and last basophilic rubricytes. For the nuclei, pixelation was also highest for senescent cells, followed by equal pixelation for polychromatophilic rubricytes and polychromatophilic erythrocytes, for which the nuclei were more pixelated than for mature erythrocytes and basophilic rubricytes (Fig. 2.7).

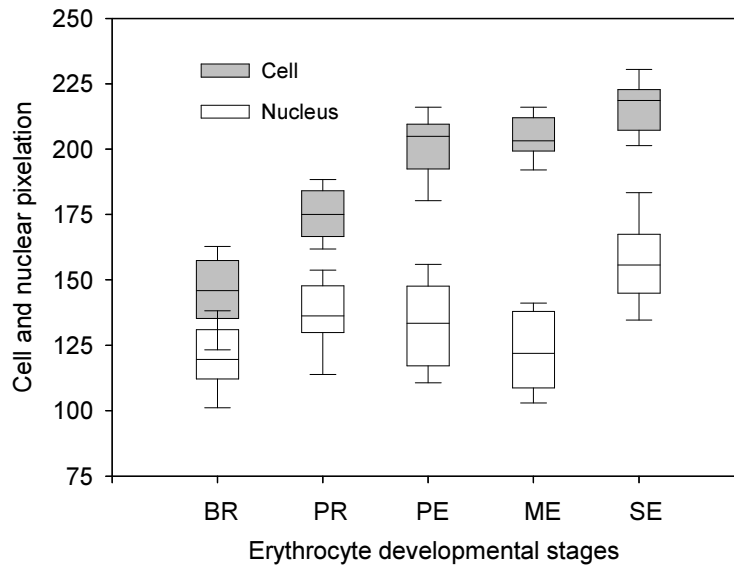


Figure 2.7 Cell and nuclear pixelation of *Homopus areolatus* for the five developmental stages (BR represents basophilic rubricytes, PR represents polychromatophilic rubricytes, PE represents polychromatophilic erythrocytes, ME represents mature erythrocytes and SE represents senile erythrocytes). The box plot indicates the median, 25% and 75% percentiles and the whiskers represent 10% and 90% percentiles. Data for cell pixelation were parametric, but were indicated as medians for consistency with other figures.

2.3.4 Description of aberrant types and features

The peripheral blood of *H. areolatus* showed a variety of aberrant erythrocyte types in low numbers. Erythrocytes that varied in shape, the poikilocytes, often had a teardrop shape (Fig. 2.8 a). Size variation of erythrocytes or anisocytosis (Fig. 2.8 b) was evident in some individuals, with both abnormally large mature erythrocytes, referred to as macrocytes (Fig. 2.8 c), and unusually small erythrocytes, known as microcytes (Fig. 2.8 d), being present. The blood also contained erythrocytes without nuclei known as erythroplastids (Fig. 2.8 e). Erythrocytes with intracellular vacuoles (Fig. 2.8 f,g) and basophilic inclusions (Fig. 2.8 h,i) were relatively common in the blood smears. There was also evidence of cell division, presented either as amitosis (Fig. 2.8 j,k) or as different mitotic stages, such as prophase (Fig. 2.8 l).

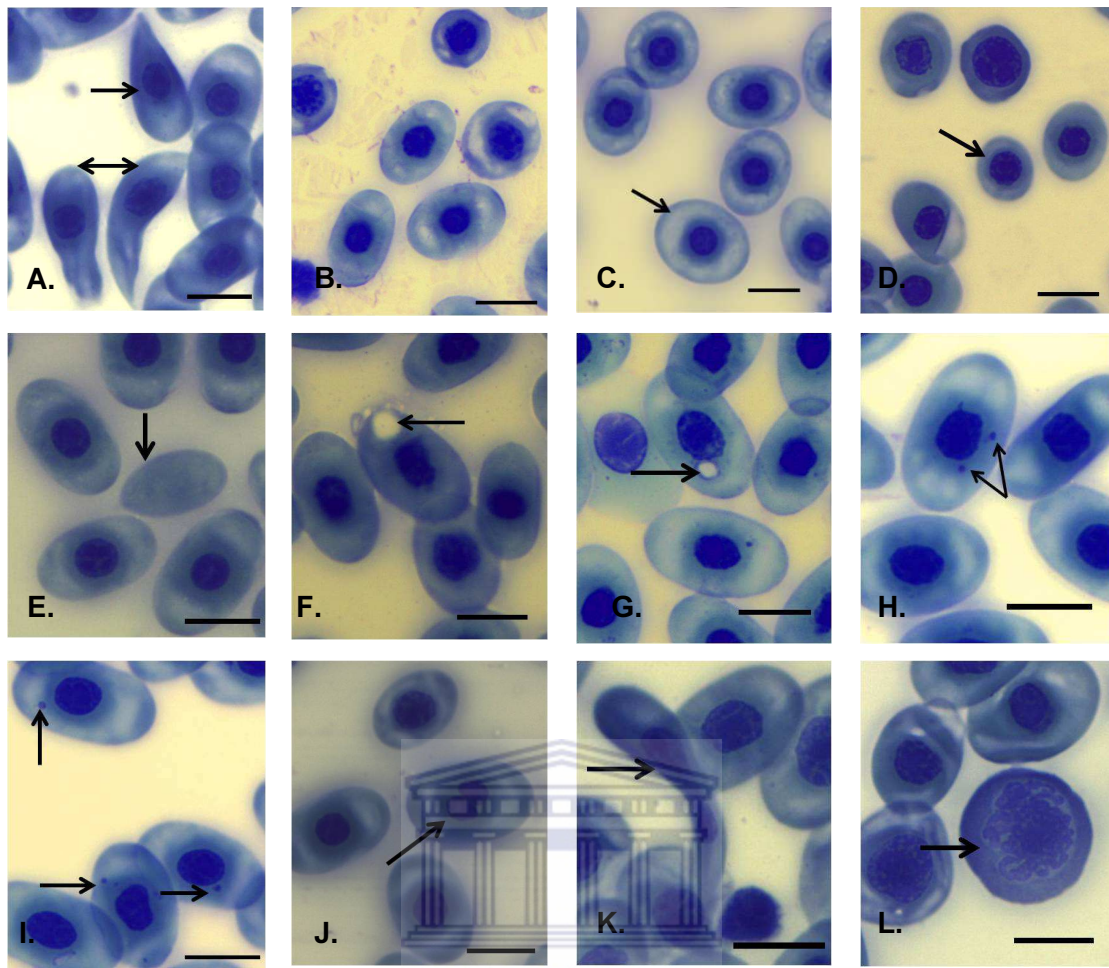


Figure 2.8 Aberrant erythrocytes of *Homopus areolatus* showing (a) poikilocytosis, (b) anisocytosis, (c) a macrocyte, (d) a microcyte (e) an erythroplastid (f & g) cytoplasmic vacuoles, (h & i) basophilic inclusions in the cytoplasm, (j & k) nuclear division in amitosis, and (l) chromatin unravelling during the mitotic prophase stage. The scale represents 10 μm at 1000 x magnification.

2.3.5 Prevalence of erythrocyte types and features

I did not detect parasites in the bloodsmear of any *H. areolatus* but recorded the other features. The data did not meet the assumptions for two-way ANOVAs, consequently I used one-way ANOVAs to assess cohort and season separately. Cohort did not affect any feature or erythrocyte type ($H_2 = 0.12$ to 3.56 , $P = 0.17$ to 0.94). Rubricytes were relatively prevalent in *H. areolatus* blood (median, 25%, 75%: 2.0, 1.0, 2.0) and were more abundant in winter than in spring and autumn ($H_3 = 15.80$, $P = 0.0012$). Overall, polychromatophilic erythrocytes were less abundant (median, 25%, 75%: 1.0, 1.0, 2.0) and were more prevalent during winter, spring and summer than in autumn ($H_3 = 15.54$, $P = 0.0014$). To strengthen my results, I ran a second ANOVA for combined data of immature erythrocytes (rubricytes + polychromatophils) and the results

showed that immature erythrocytes were more prevalent in winter, spring and summer than in autumn ($F_{3,78} = 6.08$, $P = 0.0009$). The abundance for senescent erythrocytes was low (median, 25%, 75%: 1.0, 1.0, 2.0) and these cells were more prevalent in autumn than in spring and summer ($H_3 = 37.33$, $P < 0.0001$; Table 2.2).

I also combined scores for the three developmental stages (all cohorts combined) to gain additional insights into their seasonal prevalence. For all seasons combined, the score was higher for rubricytes than for polychromatophils and senile erythrocytes ($H_2 = 11.64$, $P = 0.0030$). In autumn, senile erythrocytes had the highest score, followed by rubricytes and then polychromatophilic erythrocytes ($H_2 = 23.12$, $P < 0.0001$). This situation changed in winter, when rubricytes were more prevalent than polychromatophilic and senile erythrocytes ($H_2 = 14.42$, $P = 0.0007$). In both spring and summer, the scores for rubricytes and polychromatophilic erythrocytes did not differ, but both cell types were more prevalent than senile erythrocytes ($H_2 > 12.99$, $P < 0.0016$).

The prevalence for erythroplastids (median, 25%, 75%: 0, 0, 1), microcytes (median, 25%, 75%: 0, 0, 0), cytoplasmic inclusions (median, 25%, 75%: 0, 0, 1) and cytoplasmic vacuoles (median, 25%, 75%: 0, 0, 1) was low but I detected a somewhat higher prevalence for poikilocytosis (median, 25%, 75%: 1, 1, 1) and macrocytosis (median, 25%, 75%: 1, 0, 1). Season did not influence the incidence of poikilocytosis ($P = 0.230$) but affected the prevalence of erythroplastids, macrocytosis and microcytosis, as well as cytoplasmic vacuoles and inclusion bodies ($H_3 > 9.94$, $P < 0.019$). The incidence for erythroplastids was higher in spring and summer than in autumn. Macrocytosis was more prevalent in summer than in autumn whereas microcytosis did not show a significant post hoc difference among seasons. Cytoplasmic inclusions had a higher prevalence in autumn than in winter, whereas cytoplasmic vacuoles were more prevalent in autumn and winter than in summer and spring.

2.4 DISCUSSION

2.4.1 Erythrocyte morphology and development

The peripheral blood of *H. areolatus* contained five developmental stages from basophilic rubricytes through mature to senescent erythrocytes. Overall, development up to maturity involved that the cells increased in size, that the nuclear to cellular ratio decreased, that cell shape changed from round to oval, that cytoplasmic staining

changed from basophilic to eosinophilic, and that nuclear chromatin became more condensed. The same pattern has been reported for several other reptile species (Mader 2000; Campbell 2004; Stacy et al. 2011; Nardini et al. 2013) although some reports indicate that cell size decreases with maturity (Bernstein 1938).

For *H. areolatus*, cell length, width and area were smallest for basophilic rubricytes. This stage also had the highest nuclear to cellular ratio, was the roundest of all developmental stages, and had the lowest pixelation, meaning that the cytoplasm stained the most basophilic of all stages. The uniform basophilic appearance of the cytoplasm can be ascribed to the abundance of RNA and the absence or low presence of haemoglobin (Quigley et al. 2014). The nucleus stained deeply basophilic (low pixelation) and the chromatin network was partially clumped, as has been indicated in other reptiles (Campbell and Ellis 2007; Campbell 2012). Continued development involved an increase in cell area up to maturity, mostly through an increase in length. Width actually decreased in the final stages of maturity, leading to a highly elongated mature erythrocyte. Although many authors note that reptile erythrocyte size increases through development (Stacy et al. 2011; Campbell 2012), I am not aware of any study that link this increase to a differential growth in cell length and width. Nuclear staining intensity decreased in the polychromatophilic rubricytes and polychromatophilic erythrocytes of *H. areolatus*, and the chromatin appeared more condensed. At these developmental stages, the paler-staining and more open regions of the euchromatin indicate active haemoglobin production (Strik et al. 2007; Stacy et al. 2011). The chromatin network became fully condensed at maturity (Campbell and Ellis 2007; Strik et al. 2007), and staining intensity of the nuclei was yet again similar to that of basophilic rubricytes. Colour changes of the cytoplasm from uniformly basophilic through polychromatophilic to uniformly acidophilic, as cells progress from immature to mature, were related to the production and accumulation of haemoglobin in the cytoplasm (Quigley et al. 2014).

Nuclear size and shape changes of *H. areolatus* erythrocytes were less pronounced than for the cells. The large nucleus of immature cells shrank only in the final developmental stages towards maturity, first through a decrease in width, then by a decrease in length. Consequently, nuclear shape remained relatively round through all developmental stages up to maturity. The nuclear to cellular ratio of *H. areolatus* decreased progressively from basophilic rubricytes to mature erythrocytes, as has been reported for other reptiles (Strik et al. 2007; Stacy et al. 2011; Campbell 2012; Walton 2012; Nardini et al. 2013). This decreasing ratio was brought about mainly by

cytoplasmic growth, with a reduction in nuclear size becoming a factor only at the polychromatophilic erythrocyte stage. A similar observation was made by [Walton \(2012\)](#) for another endemic South African tortoise, *P. geometricus*.

Cell shape changed again when mature erythrocytes became senescent, and here an increase in width rather than a change in length resulted in senescent cells being less elongated than mature cells. A larger size and less elongated shape of senescent erythrocytes were reported also for other reptiles ([Pienaar 1962](#); [Jacobson 2007](#); [Walton 2012](#)). Nuclear size also increased, but this was due to increases in both length and width. The weak staining of the nucleus and cytoplasm of senescent erythrocytes of *H. areolatus* corresponded to the findings of many scientists for other reptiles ([Pienaar 1962](#); [Jacobson 2007](#); [Walton 2012](#)). Senescence signifies the end of the erythrocyte life cycle, where all cell and nuclear properties begin to disintegrate ([Pienaar 1962](#); [Campbell 2012](#)) and weaker staining properties are the first signs of disintegration. Reduced staining of the cytoplasm probably signifies the decline or disintegration of haemoglobin whereas the weak, lilac nuclear staining is probably due to the disintegration of nuclear contents as the erythrocyte reaches the end of its life cycle ([Nardini et al. 2013](#)).

Although the early immature erythrocyte stages of *H. areolatus* were in general substantially smaller than mature cells, I detected a small percentage of relative large immature stages in the blood smears. This size variation may be attributable to the existence of more than one erythrocyte lineage, as proposed by [Pienaar \(1962\)](#) and [Frye \(1991\)](#). [Pienaar \(1962\)](#) believed that the lymphoid lineage is the primary pathway that gives rise to small immature erythrocytes, whereas the myeloid stem cell lineage, which produces larger immature erythrocytes, is only activated during periods of stress. [Frye \(1991\)](#) on the other hand proposed that the myeloid stem cell lineage is the primary pathway and that pluripotent thrombocytes give rise to small-sized erythrocytes during periods of acute and chronic blood loss. Although the presence of two size classes of immature erythrocytes in *H. areolatus* may indicate the existence of more than one developmental lineage, the proposals forwarded by [Pienaar \(1962\)](#) and [Frye \(1991\)](#) may not necessarily provide the explanation.

Nowadays, haematologists distinguish between primitive and definitive haemopoiesis during embryonic development. Primitive haemopoiesis gives rise mainly to erythrocytes whereas definitive haemopoiesis leads to haematopoietic stem cells that have the potential to develop in all blood cell types in various tissues ([Steward and](#)

Florian 2000; Martinez-Agosto et al. 2007; Fraser 2013). Cells of these two erythroid lineages differ morphologically, for example, primitive erythroid cells are larger than definitive erythroid cells are (Fraser 2013). Furthermore, primitive erythropoiesis could be reactivated under certain conditions (Fraser 2013), which may explain the presence of both small and large immature erythrocytes. An alternative explanation relates to the finding that in mammalian embryos, definitive erythrocytes derived from the liver are larger than are their counterparts derived from bone marrow (Fraser 2013). In reptiles, erythropoiesis can occur outside the bone marrow (Campbell 2012) and lineages from different tissues may differ morphologically. At this stage, it is not possible to conclude which pathway may explain the variation in cell size of immature erythrocytes in *H. areolatus* or if the same explanation is valid for all chelonians for which immature erythrocytes vary in size.

The main function of erythrocytes is to transport oxygen, bound to haemoglobin, to cells in the body (Dessauer 1970; Snyder and Sheafor 1999; Stik et al. 2007). Haemoglobin production starts in the basophilic rubricytes and its concentration increases with cell maturity to be highest in mature erythrocytes (Frische et al. 2001; Quigley et al. 2013). Because they have a lower haemoglobin content, immature cells are thus not as efficient as mature cells in transporting oxygen to body cells. The size and shape of mature erythrocytes influence the efficiency and rate of gaseous exchange. (Hartman and Lessler 1964). Because small cells have a larger surface to volume ratio than large cells have, small erythrocytes offer a greater rate of oxygen exchange than larger ones do (Shadkhast et al. 2010; Javanbakht et al. 2013). In the lower vertebrates, chelonians have the second largest erythrocytes, but among chelonians, terrestrial species have the smallest erythrocytes (Arikan and Çiçek 2010). The shape of mature erythrocytes of lower vertebrates such as tortoises, are elongated, whereas the immature erythrocytes are more circular (Knotková et al. 2002; Zhang et al. 2011; Campbell 2012; Walton 2012).

I did not encounter parasites in the erythrocytes of *H. areolatus* but recorded most aberrant features mentioned in the literature. *Homopus areolatus* had a low to moderate level of poikilocytosis, which may have been associated with an increased level of erythropoiesis (Pendl 2006); this deduction is supported by the relatively high incidence of immature erythrocytes in their blood. Poikilocytosis can also be considered as slide preparation artefacts (Nardini et al. 2013), but this is usually the case when the incidence is low. The presence of erythroplastids was rare in *H. areolatus*, as is generally found in reptiles, and is considered to have no clinical

significance (Campbell 1996; Stacy et al. 2011, Nardini et al. 2013). Although the prevalence of macrocytes was somewhat higher than for microcytes in *H. areolatus*, the degree of anisocytosis was relatively low. A low level of anisocytosis is seen as normal in reptiles whereas elevated levels can be indicative of a regenerative response or erythrocyte disorders (Reavill 1994; Campbell 2004; Stacy et al. 2011). I encountered low levels of intra-cytoplasmic vacuoles and basophilic inclusions in the erythrocytes of *H. areolatus*. Such structures are seen as normal in healthy chelonians and the basophilic inclusions are considered degenerated organelles (Campbell 2004; Pendl 2006; Oliveira-Júnior et al. 2009), although it may also be indicative of a regenerative response (Nardini et al. 2013).

2.4.2 Effect of cohort and season

Cohort had no effect on the prevalence of polychromasia, poikilocytosis, erythroplastids, macrocytosis, microcytosis, cytoplasmic vacuoles and inclusions, indicating that factors influencing these features applied similarly to all ages and sexes in *H. areolatus*. According to many scientists, juvenile reptiles are known to have a higher incidence of immature erythrocytes than adult reptiles have (Campbell 2004; Nardini et al. 2013), but this was not the case in *H. areolatus*.

Season, in contrast, had a significant effect on the prevalence of all erythrocyte features in *H. areolatus*, except the occurrence of poikilocytosis. This study took place in the south-western Cape, a winter rainfall region with a Mediterranean climate (van Bloemestein 2005; Walton 2012), where winter temperatures are sufficiently mild to allow ectotherms such as tortoises to be active throughout the year (Litzgus and Hopkins 2003; Walton 2012; Gillooly and Zenil-Ferguson 2014). The fluctuations in rainfall and temperature through the year influence plant growth and thus the availability of food to herbivores, such as tortoises (Joshua et al. 2010; Crucitti 2012). At Elandsberg, winter rains commenced in April to May (late autumn) and continued up to September-October. The late-autumn rains stimulate plant growth, making an abundance of food plants available by winter (Joshua et al. 2010; Walton 2012). Sufficient food remains available throughout spring, but becomes progressively scarcer through summer and reaches lowest levels in autumn (Walton 2012; M.D. Hofmeyr, pers. comm.). Fluctuations in food and water availability, together with temperature fluctuations, probably explain the effect of season on erythrocyte features.

In *H. areolatus*, the prevalence of rubricytes, the earliest immature stage detected in peripheral blood, was highest in winter. In addition, the prevalence of polychromatophilic erythrocytes, a later developmental stage, was higher in winter, spring and summer than in autumn, indicating an elevated level of erythropoiesis from winter to summer. The initial erythropoietic response may be related to either a drop in temperature or an increase in food availability. In some ectotherms, low temperatures *per se* can induce a regenerative erythropoietic response, but in these experiments, new erythrocytes remained in the liver and did not migrate to the circulation (Maekawa et al. 2012), which was not the situation in *H. areolatus*. A regenerative erythropoietic response has been reported also for ectotherms emerging from a period of limited metabolic activity such as hibernation (Pienaar 1962; Campbell 2004; Pendl 2006). Although activity levels of *H. areolatus* is limited in the dry season (M.D. Hofmeyr, pers. comm.), there is no evidence that they have low metabolic levels prior to the onset of winter. Similar to *H. areolatus*, a sympatric tortoise species, *P. geometricus*, shows a proliferation of immature erythrocytes, indicative of a regenerative erythropoietic response, in winter and spring (Walton 2012). Walton (2012) ascribed the regenerative erythropoietic response to an emergence from a period of limited metabolic activity (the dry season), but an increase in food availability in winter may equally well have stimulated erythropoiesis in both *P. geometricus* and *H. areolatus*.

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The prevalence of senescent erythrocytes was higher in autumn than it was in spring and summer. Autumn was the most stressful season for *H. areolatus* because it signified the end of an extended period where food and water were limited (M.D. Hofmeyr, pers. comm.). Consequently, one would not expect that *H. areolatus* individuals would have been in a good nutritional state at this stage. In fact, one would expect to see physiological stress and an increase of aberrant erythrocyte types and features. The latter serves as a plausible explanation for the high incidence of senescence and the increased manifestation of intra-cytoplasmic vacuolation as well as intracytoplasmic basophilic inclusions during autumn (Chansue et al. 2011; Campbell 2012). The fact that more macrocytes were indicated in summer than in autumn, may be linked to a greater percentage of fully matured erythrocytes in summer, before senescence sets in due to elevated physiological stress. Although the prevalence for erythroplastids was low in *H. areolatus*, these structures had a higher incidence in spring and summer than in autumn. Their occurrence is generally regarded as incidental but Glomski et al. (1997) postulated that erythroplastids transport oxygen more efficiently because the lack of a nucleus eliminates the oxygen requirements of that structure. This explanation may have relevance for *H. areolatus*

because the metabolic requirements of this ectotherm would increase at the higher temperatures of spring and summer (Brown et al. 2005; Keswick et al. 2006; Walton 2012).

2.5 CONCLUSION

This is the first study to describe erythrocyte morphology and the different developmental stages in peripheral blood of *H. areolatus*. I observed basophilic and polychromatophilic rubricytes as well polychromatophilic erythrocytes. In addition, mature and senile erythrocytes were observed. Pixelation and nuclear to cellular area ratios played a key role in distinguishing among the various developmental stages, and the process was facilitated by the use of imaging software. Evidence suggests immature erythrocytes within *H. areolatus* may have evolved from two distinctive lineages. However, further investigation is required to identify which lineage gave rise to either large or small immature erythrocytes in this study.

Despite the fact that erythrocyte development was not influenced by cohort, seasonal effects were however evident. Winter and spring appeared to be the seasons where erythropoiesis was most prevalent. The blood profile on the other hand indicated that autumn was the most stressful season, since it had the highest incidence of senescence, intra-cytoplasmic vacuolation and intra-cytoplasmic basophilic inclusions. Overall, it appears that during periods of high food and water availability (winter and spring), erythropoiesis increases, while erythrocytes show signs of physiological stress as food becomes progressively scarce through summer and autumn respectively.

Histological studies focussed upon erythrocyte development among chelonian species are scarce. I recommend further research to be conducted upon erythrocyte development of South African tortoises in particular as this study marks only the second of its kind.

3 ERYTHROCYTE MEASUREMENTS – SIZE AND SHAPE

3.1 INTRODUCTION

Vertebrates rely principally on aerobic pathways to supply energy for metabolism and growth (Jackson 2007; Torres et al. 2012). The oxygen required for aerobic metabolism is transported in the blood from the respiratory organs to the body tissues. When blood is oxygenated, a small proportion of oxygen is transported in the dissolved state but most oxygen molecules are bound reversibly to haemoglobin tetramers in the erythrocytes (Strik et al. 2007; Weber 2007; Jensen 2009). The protein haemoglobin consists of four subunits, giving each haemoglobin molecule the capacity to carry four oxygen molecules when fully saturated (Jensen 2009). Under high oxygen pressure, as in the lungs or gills, oxygen diffuses into the erythrocytes to saturate the haemoglobin fully, but because the binding is reversible, haemoglobin releases the oxygen molecules in the body tissues where the oxygen pressure is low (Jensen 2009). The amount of oxygen transported in the blood is directly dependent on total haemoglobin concentration (Pough 1980; Vandegriff and Olson 1984), and the size and number of erythrocytes again influence haemoglobin concentration. The size and shape of erythrocytes influence the diffusion rate of oxygen into the cells, which ultimately affects the amount of oxygen carried in the erythrocytes (Hartman and Lessler 1964).

Vertebrate erythrocytes differ substantially in size and there appears to be a negative correlation between erythrocyte size and the rate of metabolism (Starostová et al. 2013). Because metabolic rate is influenced by temperature, there is also an exponential increase in total red cell volume of vertebrate groups with the temperature at which each group functions (Gillooly and Zenil-Ferguson 2014). Total red cell volume is a function of erythrocyte size and number. Consequently, a reduction in erythrocyte size is accompanied by an increase in erythrocyte number not only among vertebrate groups, but also for species of the same genus (Frair 1977; Arıkan and Çiçek 2010; Shadkhast et al. 2010; Stacy et al. 2011; Zhang et al. 2011). Since smaller erythrocytes would have less space for haemoglobin, it seems reasonable that the number of circulating erythrocytes increases to counter a reduction in haemoglobin content. In general, amphibians have the largest erythrocytes, followed by fish and reptiles, with the two endothermic vertebrates, birds and mammals, having the smallest erythrocytes (Uğurtaş et al. 2003; Javanbakht et al. 2013). Mammals are the only vertebrates with enucleated erythrocytes (Strik et al. 2007; Stacy et al. 2011),

which may represent an evolutionary compromise to create more space for haemoglobin in the small erythrocytes and thus improve the efficacy of oxygen transportation (Glomski et al. (1997).

Apart from the fact that erythrocyte size has a direct effect on haemoglobin content, size also affects the rate of gas exchange across the erythrocyte membrane. Small erythrocytes have a higher surface area to volume (SA:V) ratio than large erythrocytes have, creating a greater potential for efficient gaseous exchange in small erythrocytes (Wojtaszek and Adamowicz 2003; Gregory et al. 2009; Grenat et al. 2009; Motlagh et al. 2010; Hatami et al. 2014). The SA:V effect seems to be of particular importance in oxygen uptake and of lesser importance in oxygen release (Vandegriff and Olson 1984). Shape also influences the SA:V ratio of objects. A sphere has the lowest SA:V ratio of all shapes and the ratio increases when the object elongates (Hartman and Lessler 1964). Consequently, ellipsoidal erythrocytes have a higher SA:V ratio, and more efficient gas exchange, than spherical erythrocytes have (Hartman and Lessler 1964).

Because erythrocytes play such a central role in vertebrate physiology, it is important to understand their normal morphological variations to be able to identify changes brought about by physiological stress and disease (Paul et al. 2008; Zhang et al. 2011). For instance, *P. geometricus* experienced haemodilution during winter and spring characterised by large mature erythrocytes, while mature erythrocytes were smaller during drier months amongst cohorts (Walton 2012).

Despite the fact that South Africa is rich in tortoise diversity, haematological studies are limited. Essentially, haematological baseline values have been established for only one endemic tortoise, *Psammobates geometricus* (Walton 2012). The aim of this chapter was to establish baseline values for the erythrocyte morphology of *H. areolatus* and to examine how season and cohort influence erythrocyte size and shape. Specific objectives were to (1) characterize erythrocyte size, shape and colour, (2) assess how cohort influences cell and nuclear parameters and link dissimilarities to different requirements of age groups and sexes, and (3) evaluate the effect of seasonal environmental changes on cell and nuclear parameters and assess how differences tie in with the physiological requirements of the cohorts.

3.2 MATERIALS AND METHODS

See Chapter 2 for a description of field procedures and the staining of blood smears.

3.2.1 Histological evaluation of mature erythrocytes

To perform histological evaluation on mature erythrocytes of all samples (males, females and juveniles over four seasons), I used a Leica DM 500 digital photomicroscope (SMM Instruments Pty, Ltd) with a 10x eyepiece and a 40x objective to give 400x magnification followed by a 100x objective to give 1000x magnification under immersion oil. The microscope was linked with Leica LAS software version 1.8.0 (Leica Microsystems Ltd., Heerbrugg, Switzerland) to a Leica ICC50 camera (Wetzlar, Germany). Using the meandering technique at the 40x objective, I switched to the 100x objective whenever I encountered a field of view with 10 typical mature erythrocytes to photograph the cells under immersion oil. The photographs were saved as jpeg files at 2048 x 1536 pixels for a permanent record. I recorded 10 images per slide in order to perform cell and nuclear measurements of 100 erythrocytes per individual tortoise.

I measured and analysed the photographs of mature erythrocyte with Nikon NIS Elements imaging software (Basic Research version 3.10 Inc., Nikon Instruments, Europe B.V., AS Amstelveen, The Netherlands). The parameters that were measured automatically for both the cell and nucleus included area (surface area in μm^2), length (the longest axis in μm), width (calculated from area/length in μm), elongation (determined from a set of Feret's diameters between 0 and 180 degrees with 10 degree angle intervals as MaxFeret/MinFeret), and mean intensity (the statistical mean of intensity values of pixels). These parameter definitions are in accordance with the glossary of Nikon NIS Elements.

Before taking measurements, pixel size was manually calibrated to a micrometer scale so that 1 pixel correspond to 0.064 μm for images captured at 1000x magnification. Each image was then optimised to reduce staining artifacts and to improve cellular and nuclear boundaries. The Nikon NIS Elements system uses pixelation to distinguish between light and dark images, therefore I used the contrast function to distinguish the nucleus from the cytoplasm. After contrast adjustments, the nucleus was darker than the cytoplasm. I adjusted the white saturation intensity to wash out staining artifacts that might be present in the background on the slide. The "auto detect" threshold function (from the binary toolbar of NIS system) was then used

to obtain the best measurement to further resolve nuclear and cellular boundaries. Subsequently, I used the “erode” or “open” function of NIS to select a cell and match cellular and nuclear boundaries with threshold values. NIS Elements digitally computerized measurements to an accuracy of 0.01 μm and exported measurements to a Microsoft Excel 2007 spreadsheet for further analyses.

3.2.2 Data and statistical analysis

Data for mature erythrocytes of all individuals were combined in a Microsoft Excel 2007 spreadsheet. For all statistical evaluations, I used SigmaStat (SPSS Inc., Chicago, U.S.A. version 2.03). The objective was to test for differences in cell and nuclear dimensions and colour intensity among cohorts and seasons, but two-way ANOVAs were not possible because the data were not parametric. Consequently, I evaluated the effects of season and cohort separately through one-way ANOVAs (F statistic) for parametric data and Kruskal-Wallis ANOVAs on ranks (H statistic) for non-parametric data. For post hoc comparisons, Student-Newman-Keuls were used for parametric one-way ANOVAs, whereas Dunn's post hoc comparisons were used for Kruskal-Wallis ANOVAs. I first combined all seasons to test for cohort differences overall and subsequently tested for cohort differences within each season. Similarly, I combined all cohorts to first evaluate the effect of season overall and then tested for seasonal effects within each cohort.

Because erythrocyte cell areas varied widely, I divided the erythrocytes into six size categories to evaluate differences in frequency distributions of size classes. Size class 1 represented erythrocytes with cell areas $\leq 120 \mu\text{m}^2$, size classes 2, 3, 4 and 5, respectively, represented erythrocytes cell areas from 120.1 to 130.0 μm^2 , 130.1 to 140.0 μm^2 , 140.1 to 150.0 μm^2 and 150.1 to 160.0 μm^2 , and size class 6 represented erythrocytes $> 160 \mu\text{m}^2$. Since the frequencies of small and large erythrocytes may reflect important physiological states, I also divided the data into fewer size classes in order to contrast the smallest size class against the remainder (≤ 120 against $> 120 \mu\text{m}^2$) and the largest size class against the remainder (> 160 against $\leq 160 \mu\text{m}^2$).

I used Chi-square tests to test if erythrocyte size class frequencies, and small or large erythrocytes versus the remainder, differed among cohort and season, and used a Yates correction for continuity when required. I first tested if size class frequencies differed among seasons within each cohort, and subsequently tested for differences between specific seasons of each cohort. Similarly, I tested if size class frequencies differed among cohorts within each season, and subsequently tested for differences

between specific cohorts for each season. Because multiple (30) tests were done to evaluate the effects of season and cohort on frequency distributions of erythrocyte size classes, and frequencies of small or large cells versus the remainder, I applied sequential Bonferroni corrections to each family of tests.

3.3 RESULTS

3.3.1 Erythrocyte cell and nucleus size

In order to simplify reporting of my results, I will use symbols (smaller than, <; greater than, >; equal, =) and abbreviations (male, M; female, F; juvenile, J; spring, Sp; summer, Su; autumn, Au; winter, Wi) to summarise the outcome of comparisons among cohorts and seasons.

The overall pattern (combined seasons) for cohort comparisons of erythrocyte cell area (Table 3.1) was M>J>F whereas it was M>F>J for cell length and J>M>F for cell width ($H_2 > 110.2$, $P < 0.0001$). The pattern for nuclear area, length and width, respectively, was J>F=M, J>M>F and J>F>M ($H_2 > 57.1$ $P < 0.0001$). The results for seasonal comparisons (Table 3.2; combined cohorts) of erythrocyte size were that cell area and width showed a similar pattern, Wi>Au>Sp=Su, whereas the pattern for cell length was Au>Wi>Sp>Su ($H_3 > 471.6$, $P < 0.0001$). The dimensions for both nuclear area and width decreased in the sequence Wi>Sp>Au>Su. The pattern was similar for nuclear length, except that it did not differ in spring and autumn ($H_3 > 491.4$ $P < 0.0001$; Wi>Sp=Au>Su).

Table 3.1 Measurements (medians, 25% and 75% percentiles) for erythrocyte cell and nuclear area, length and width of female, male and juvenile *Homopus areolatus* for all four seasons combined, with area in μm^2 , and length and width in μm .

Parameter	Female	Male	Juvenile
Cell area	135.0, 120.9, 151.2	142.3, 126.6, 159.9	136.4, 122.2, 154.8
Cell length	16.6, 15.5, 17.6	17.0, 15.9, 18.1	16.2, 15.1, 17.3
Cell width	8.2, 7.5, 9.0	8.4, 7.7, 9.1	8.5, 7.8, 9.3
Nuclear area	21.0, 18.2, 24.0	20.9, 18.2, 24.2	22.2, 18.8, 25.9
Nuclear length	5.7, 5.3, 6.1	5.8, 5.4, 6.3	5.9, 5.4, 6.3
Nuclear width	3.7, 3.4, 3.9	3.6, 3.4, 3.9	3.8, 3.4, 4.2

Table 3.2 Seasonal measurements (medians, 25% and 75% percentiles) for erythrocyte cell and nuclear area (A), length (L) and width (W) of *Homopus areolatus* cohorts combined, with area in μm^2 , and length and width in μm .

	Spring	Summer	Autumn	Winter
Cell				
A	131.5, 117.4, 146.6	130.3, 117.6, 144.7	144.6, 128.4, 163.1	146.1, 132.1, 161.1
L	16.4, 15.3, 17.5	16.2, 15.2, 17.2	17.2, 16.0, 18.4	16.9, 15.9, 17.8
W	8.0, 7.4, 8.8	8.0, 7.4, 8.8	8.5, 7.7, 9.2	8.7, 8.1, 9.4
Nucleus				
A	21.6, 18.6, 24.9	18.9, 16.3, 22.7	20.8, 18.6, 23.6	22.8, 20.2, 25.5
L	5.8, 5.4, 6.3	5.5, 5.0, 4.0	5.8, 5.4, 6.2	5.9, 5.5, 6.3
W	3.7, 3.4, 4.0	3.5, 3.2, 3.8	3.6, 3.4, 3.9	3.8, 3.6, 4.1

Erythrocyte area (Fig. 3.1) of females was largest during winter and smallest during summer ($W_i > A_u > S_p > S_u$) whereas seasonal differences in erythrocyte area were $W_i > A_u > S_u > S_p$ for males and $W_i = A_u > S_p > S_u$ in juveniles ($H_3 > 159.7$, $P < 0.0001$). Seasonal changes in cell length were $W_i > S_p > S_u$, also with $A_u > S_u$ in females and $A_u > W_i > S_p = S_u$ in males. In juveniles autumn cell length was greater than in all other seasons and winter length was greater than in spring ($H_3 > 113.8$, $P < 0.0001$). Erythrocyte widths also differed among seasons for all cohorts ($H_3 > 183.0$, $P < 0.0001$): the pattern for females, males and juveniles respectively was $W_i > A_u > S_u = S_p$, $W_i > A_u > S_u > S_p$ and $W_i > S_p = A_u > S_u$.

Within season comparisons of erythrocyte size among cohorts showed significant differences. For cell area, cohort sequence differed in each season ($H_2 > 11.0$, $P < 0.0003$): in spring, $J > M$; in summer, $M > F > J$; in autumn, $M > J > F$; in winter, $M > J = F$ (Fig. 3.1). For cell length, results were similar in summer and winter ($M > F > J$) but cohort sequence differed in spring ($M = F > J$) and autumn ($M > J > F$) from the other seasons ($H_2 > 23.8$, $P < 0.0001$). Cell width measurements also differed ($H_2 > 12.4$, $P < 0.002$) but the sequences among cohorts corresponded in autumn and winter ($J = M > F$) whereas cohort sequence in spring was $J > F > M$ and in summer was $M > F > J$.

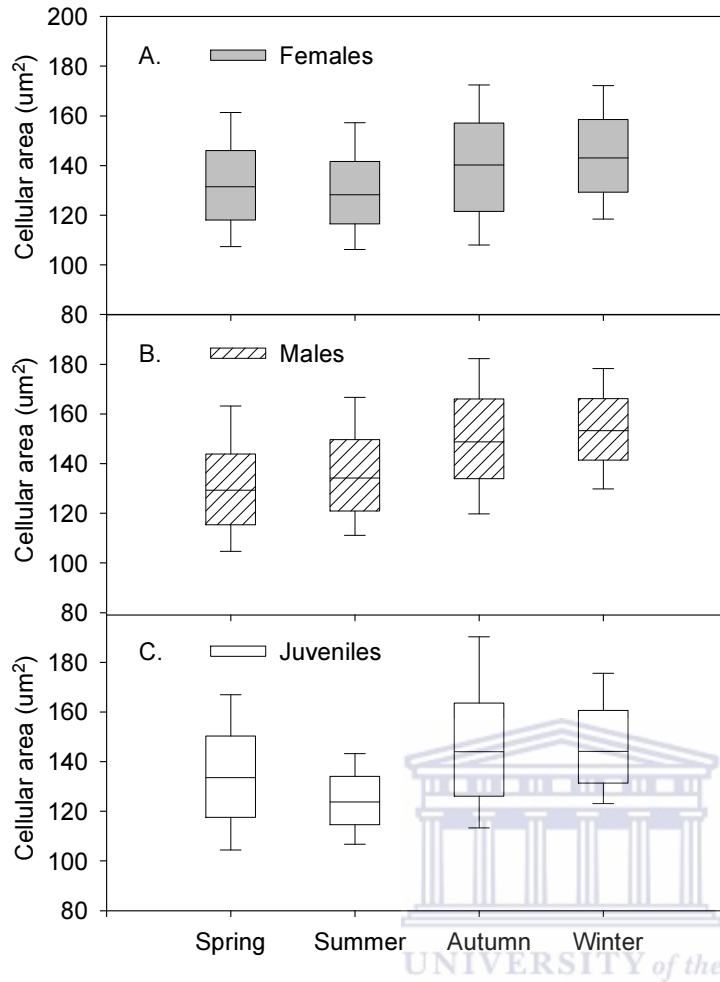


Figure 3.1 Representation of cell area (μm^2) for female (a), male (b), and juvenile (c) *Homopus areolatus* in each of four seasons. The box plot indicates the median, 25% and 75% percentiles and the whiskers represent 10% and 90% percentiles.

Nuclear area, length and width changed with season in all cohorts ($H_3 > 188.7$, $P < 0.0001$). The pattern for area was $Wi=Au>Sp>Su$ for females, $Wi>Sp>Au>Su$ for males and $Wi=Sp>Su>Au$ for juveniles (Fig. 3.2). Nuclear length in females was equally high in autumn and winter ($Au=Wi>Sp>Su$) whereas it was equally high in spring and winter for juveniles ($Sp=Wi>Su>Au$). For males, nuclear length was high in winter and spring ($Wi>Au>Su$, and $Sp>Su$), but spring lengths did not differ from either winter or autumn lengths. Nuclear widths were greatest in winter for all cohorts, but there were other differences: for females, $Wi>Au>Sp>Su$; for males, $Wi>Sp>Au>Su$; for juveniles, $Wi>Sp>Su>Au$.

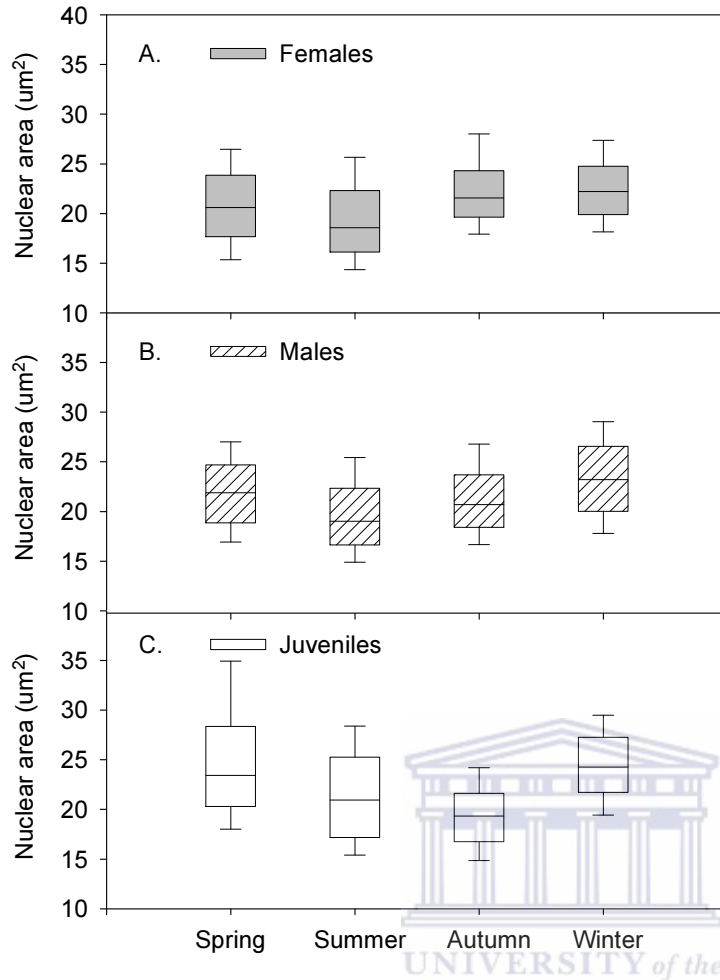


Figure 3.2 Representation of nuclear area (μm^2) for female (a), male (b), and juvenile (c) *Homopus areolatus* within each of four seasons. The box plot indicates the median, 25% and 75% percentiles and the whiskers represent 10% and 90% percentiles.

Nuclear measurements also differed among cohorts. The sequence of cohort values for nuclear area was similar in spring and winter ($J > M > F$) whereas it was $J > M = F$ in summer and $F > M > J$ in autumn ($H_2 > 24.9$, $P < 0.0001$; Fig. 3.2). Cohort sequences for nuclear length were $J > M > F$ in spring, $J > M = F$ in summer, $F = M > J$ in autumn, and $J = M > F$ in winter ($H_2 > 27.8$, $P < 0.0001$). Cohort comparisons also differed for nuclear width ($H_2 > 104.6$, $P < 0.00001$): $J > M = F$ in spring; $F > M > J$ in autumn; $J > M > F$ in winter. The summer width difference among cohorts was small ($J > F$; $H_2 = 8.8$, $P = 0.012$) but the P -value still passed the sequential Bonferroni test.

3.3.2 Erythrocyte size classes

The frequency distribution of the erythrocyte size classes differed significantly among season for females, males and juveniles ($\chi^2_{15} > 174.8$, $P < 0.0001$; Fig. 3.3). When

doing pair-wise comparisons between individual seasons for females, the distributions differed between all seasons ($\chi^2_5 > 50.1$, $P < 0.0001$) except between spring and summer ($P = 0.015$). The pair-wise comparisons for males ($\chi^2_5 > 21.2$, $P < 0.0007$) and juveniles ($\chi^2_5 > 25.1$, $P < 0.00013$) showed differences between all seasons (Fig. 3.3). Cohort comparisons showed differences in summer, autumn and winter ($\chi^2_{10} > 72.7$, $P < 0.0001$) but not in spring ($P = 0.017$). Individual cohort comparisons within seasons showed that all cohorts differed in summer, whereas in autumn and winter, males differed from females and juveniles but there was no difference between juveniles and females ($\chi^2_5 > 21.0$, $P < 0.0008$).

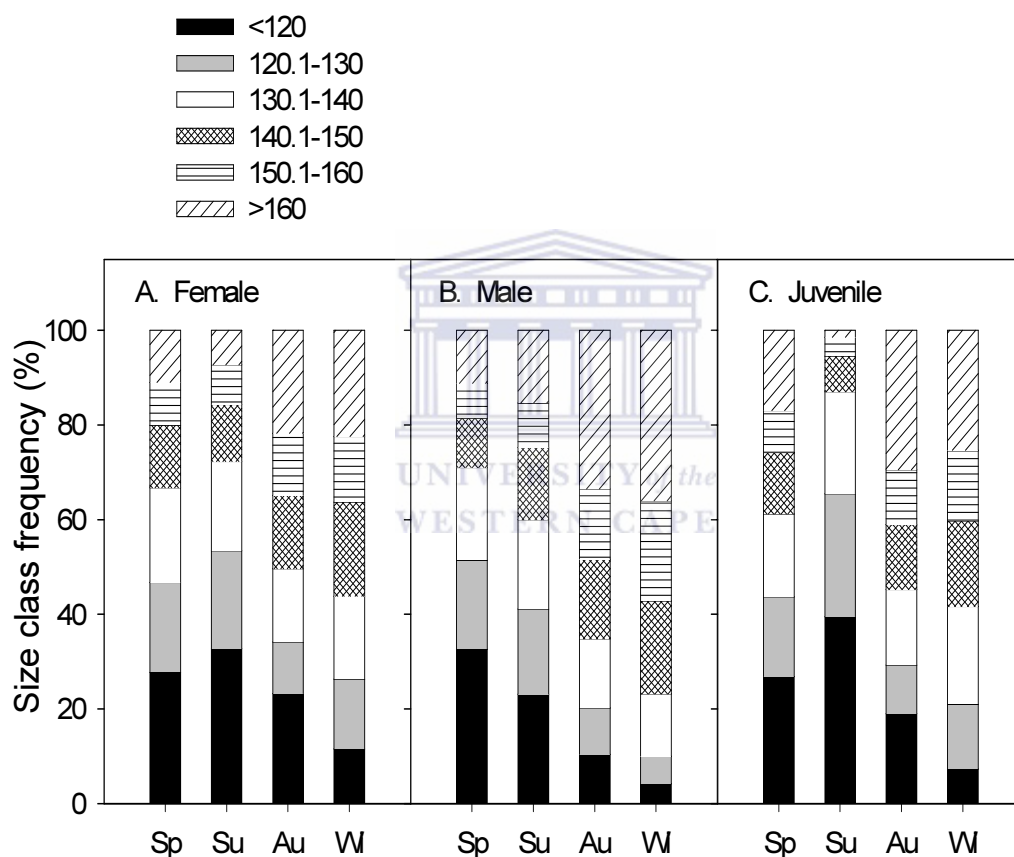


Figure 3.3 Seasonal variation in erythrocyte size classes frequency (%) of *Homopus areolatus* cohorts. Spring is Sp, summer is Su, autumn is Au and winter is Wi.

When I evaluated the frequencies of the smallest erythrocyte size class ($<120 \mu\text{m}^2$), I found that season influenced the frequencies for males, females and juveniles ($\chi^2_3 > 81.9$, $P < 0.0001$). Comparisons between individual seasons showed that the seasonal pattern of small erythrocytes for males was $\text{Sp} > \text{Su} > \text{Au} > \text{Wi}$ ($\chi^2_1 > 13.6$, $P < 0.0002$), for females $\text{Su} > \text{Au} > \text{Wi}$ and $\text{Sp} > \text{Wi}$ ($\chi^2_1 > 19.0$, $P < 0.0001$) and for juveniles $\text{Su} > \text{Sp} = \text{Au} > \text{Wi}$ ($\chi^2_1 > 10.8$, $P < 0.001$ Fig. 3.4). Within season comparisons, showed

that the cohorts differed in all seasons but spring ($X^2_2 > 23.9$, $P < 0.0001$). In summer and autumn $F=J>M$, while in winter females had a higher frequency of small cells than males had but there was no difference between females and juveniles or between males and juveniles ($X^2_1 > 18.1$, $P < 0.0001$; Fig. 3.4).

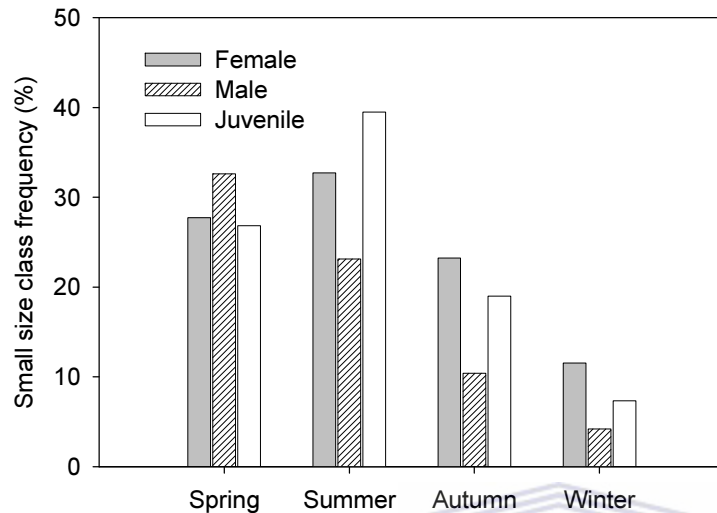


Figure 3.4 Seasonal fluctuations in the frequency (%) of small erythrocytes (<120 μm^2) of *Homopus areolatus* females, males and juveniles.

When the the largest erythrocyte size class (>160 μm^2) was considered, the frequencies were influenced by season ($X^2_3 > 74.2$, $P < 0.0001$) and by cohort ($X^2_2 > 13.9$, $P < 0.001$). Females and juveniles had the same seasonal pattern of large erythrocyte frequencies ($Au=Wi>Sp>Su$; Fig. 3.5) whereas the pattern for males was $Au=Wi>Sp=Su$ ($X^2_1 > 8.1$, $P < 0.0043$). Large erythrocyte frequencies differed among cohorts such that in spring $J>F$, in summer $M>F>J$, in autumn $M=J>F$ and in winter $M>J=F$ ($X^2_1 > 8.2$, $P < 0.004$; Fig. 3.5).

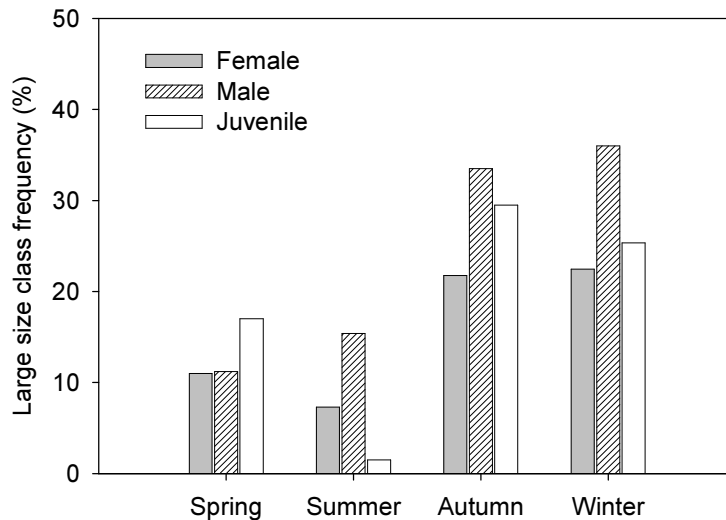


Figure 3.5 Seasonal fluctuations in the frequencies (%) of large erythrocytes (>160 μm^2) of *Homopus areolatus* females (F), males (M) and juveniles (J).

3.3.3 Nuclear to cellular area ratio

The ratio of nuclear to cellular area differed among cohorts (J>F>M; $H_2 = 140.7$, $P < 0.0001$) for combined seasons, and among seasons (Sp>Wi>Su=Au; $H_3 = 390.5$, $P < 0.0001$) for combined cohorts. When comparing seasonal effects within cohorts, nuclear to cellular ratio was Au=Sp=Wi>Su for females, Sp>Wi>Su=Au for males, and Sp>Su=Wi>Au for juveniles ($H_3 > 62.5$, $P < 0.0001$; Table 3.3). For cohort comparisons within season, the nuclear to cellular ratio differed among cohort in all seasons ($H_2 > 40.1$, $P < 0.0001$). The relationship among cohorts was J>M>F in spring, J>F>M in summer, F>M>J in autumn and J>F=M in winter (Table 3.3).

Table 3.3 Nuclear to cellular area ratio of *Homopus areolatus* giving the medians with 25% and 75% percentiles across four seasons for each cohort.

	Spring	Summer	Autumn	Winter
Female	0.16, 0.13, 0.18	0.15, 0.12, 0.17	0.16, 0.13, 0.19	0.16, 0.14, 0.18
Male	0.17, 0.15, 0.19	0.14, 0.12, 0.16	0.14, 0.12, 0.16	0.15, 0.13, 0.17
Juvenile	0.18, 0.15, 0.22	0.17, 0.14, 0.21	0.13, 0.11, 0.15	0.17, 0.15, 0.19

3.3.4 Erythrocyte shape and colour

Overall, cell shape (elongation) of *H. areolatus* erythrocytes differed among cohorts (M>F>J; $H_2 = 210.0$, $P < 0.0001$) and among seasons (Au=Sp>Su>Wi; $H_3 = 195.3$, $P < 0.0001$). Elongation of female cells was high in spring (Sp>Su=Au=Wi) and the

seasonal pattern for males and juveniles, respectively, was $Sp=Au>Su>Wi$ and $Su=Au>Sp>Wi$ ($H_3 > 117.4$, $P < 0.0001$; Table 3.4). Erythrocyte elongation did not differ among cohort in summer ($P = 0.052$), but differed in the other seasons ($H_2 > 75.1$, $P < 0.0001$): elongation was $M=F>J$ in spring and winter and $M>F=J$ in autumn (Table 3.4).

Table 3.4 Seasonal changes in erythrocyte cell and nuclear elongation of female (F), male (M) and juvenile (J) *Homopus areolatus* as medians with 25% and 75% percentiles in brackets.

	Spring	Summer	Autumn	Winter
Cell				
F	1.66 (1.49, 1.81)	1.56 (1.35, 1.80)	1.57 (1.39, 1.74)	1.54 (1.38, 1.71)
M	1.68 (1.50, 1.86)	1.55 (1.37, 1.74)	1.65 (1.52, 1.79)	1.51 (1.39, 1.65)
J	1.39 (1.25, 1.66)	1.59 (1.47, 1.72)	1.55 (1.45, 1.67)	1.37 (1.24, 1.52)
Nucleus				
F	1.19 (1.15, 1.25)	1.17 (1.13, 1.25)	1.19 (1.13, 1.27)	1.17 (1.13, 1.23)
M	1.23 (1.17, 1.31)	1.17 (1.12, 1.25)	1.23 (1.15, 1.34)	1.19 (1.13, 1.26)
J	1.16 (1.13, 1.22)	1.19 (1.13, 1.32)	1.18 (1.13, 1.27)	1.13 (1.10, 1.18)

The general pattern for nuclear elongation was $M>F>J$ ($H_2 = 145.3$, $P < 0.0001$) and $Au>Sp>Su>Wi$ ($H_3 = 160.7$, $P < 0.0001$). The elongation of erythrocyte nuclei differed among season for all cohorts ($H_3 > 33.8$, $P < 0.0001$). Nuclei of females and males were most elongated in spring and autumn: for females, $Sp>Su=Wi$ and $Au>Wi$; and for males, $Sp=Au>Wi=Su$. Erythrocyte nuclei in juveniles were most elongated in summer ($Su>Sp>Wi$ and $Au>Wi$). There were differences among cohorts in nuclear elongation within each season ($H_2 > 9.3$, $P < 0.0093$). Cohort sequences in winter and spring were $M>F>J$, whereas it was $J>M$ in summer and $M>F=J$ in autumn (Table 3.4).

Pixelation of erythrocytes and their nuclei differed overall among cohorts ($H_2 > 35.8$, $P < 0.0001$) and among seasons ($H_3 > 787.3$, $P < 0.0001$). For the cells, the results indicated $M>F>J$ and $Sp>Su>Au>Wi$, whereas the results for the nuclei were $F>M=J$ and $Sp>Su=Au>Wi$ (Fig. 3.6). There were differences within cohorts for cell pixelation ($H_2 > 97.6$, $P < 0.0001$) and the seasonal patterns were $Sp>Su>Au>Wi$ for females and males, and $Au=Sp>Su>Wi$ for juveniles. Within season differences among cohorts

were $M=F>J$ in spring, $M>F>J$ in summer, $M=J>F$ in autumn, and $M=F>J$ in winter ($H_2 > 27.3$, $P < 0.0001$). Seasonal patterns of pixelation for the erythrocyte nuclei were different for each cohort group ($H_3 > 131.7$, $P < 0.0001$): it was $Sp>Au=Su>Wi$ in females, $Sp>Su>Au>Wi$ in males, and $Sp>$ all seasons and $Au>Wi$ for juveniles. Nuclear pixelation also differed within each season for cohorts ($H_2 > 13.3$, $P < 0.0012$). Cohort differences were $M=F>J$ in spring, $M>F>J$ in summer and were $F>J=M$ in autumn and winter (Fig. 3.6).

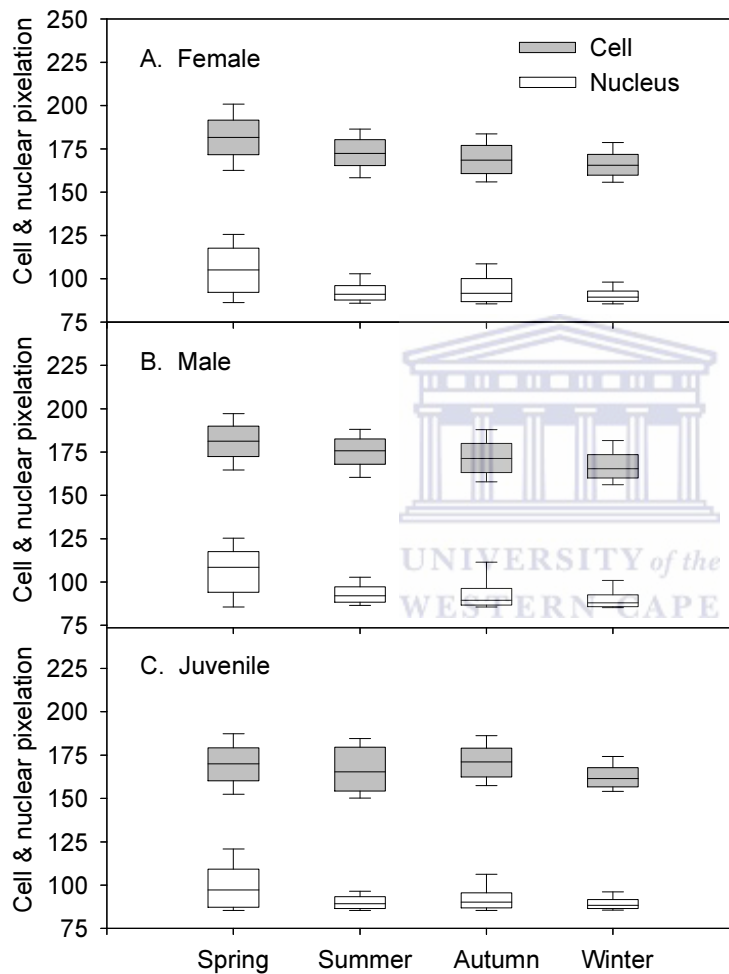


Figure 3.6 Cell and nuclear pixelation for female (a), male (b), and juvenile (c) *Homopus areolatus* over four seasons. The box plot indicates the median, 25% and 75% percentiles and the whiskers represent 10% and 90% percentiles.

3.4 DISCUSSION

3.4.1 Dimensions of mature erythrocytes of *H. areolatus*

Homopus areolatus have oval to elliptical mature erythrocytes with centrally located oval to round nuclei. The cytoplasm of mature erythrocytes has a homogenous, light

blue colour whereas the nucleus stains darkly blue (see Chapter 2 for a detailed description). This basic morphology is similar to what has been described for several other chelonians (Knotková et al. 2002; Walton et al. 2012; Javanbakht et al. 2013; Nardini et al. 2013).

Pienaar (1962) studied the haematology of South African reptiles and included one male *H. areolatus*, collected in autumn, in his study. He reported that erythrocyte length (L) varied between 16.5 and 20.5 μm and width (W) between 8.0 and 9.4 μm . In order to calculate area (A) from these measurements, I used a standard formula [$A=(L*W*\pi)/4$; Metin et al. 2008; Javanbakht et al. 2013] and found that area ranged between 103.7 and 151.4 μm^2 . These values of Pienaar correspond closely to erythrocyte dimensions of autumn males in this study where medians were 17.7 μm for length, 8.9 μm for width, and 129.3 μm^2 for area.

A comparison of erythrocyte size of *H. areolatus* with the literature is not straightforward because few studies indicate in which season the samples were taken and some do not even mention the age or sex of the animals. Furthermore, literature values for a particular species can vary substantially, as for *Testudo graeca*, for which mean erythrocyte area has been reported respectively as 67.2 μm^2 (Javanbakht et al. 2013), 139.4 μm^2 (Tosunoğlu et al. 2005) and 163 μm^2 (Uğurtaş et al. 2003). For comparative purposes, I combined seasons and cohorts for *H. areolatus* to provide mean values for erythrocyte area (139.6 μm^2), length (16.7 μm) and width (8.4 μm). It appears that *H. areolatus* erythrocytes have an intermediated size among chelonians, with several species having smaller (e.g., *Pelomedusa subrufa*, $A=98.1 \mu\text{m}^2$; Pienaar 1962) or larger (e.g., *Testudo hermanni*, $A=174.9 \mu\text{m}^2$; Uğurtaş et al. 2003) erythrocytes. Species closely related to *H. areolarus* seem to have erythrocytes of a similar size, with a mean area of 144.9 μm^2 (L=17.4 μm and W=10.6 μm) for *Stigmochelys (Geochelone) pardalis* (Pienaar 1962) and 141.37 μm^2 (L=18.0 μm and W=10.0 μm) for *Psammobates geometricus* (Bernstein 1938).

Apart from size, a comparison of cell elongation seems relevant because the degree of elongation influences how much oxygen diffuses into erythrocytes. I used mean length and width measurements from the literature to obtain an elongation factor as length divided by width. *Homopus areolatus* erythrocytes appear to be elongated (L/W=1.99) compared to many other species, for which elongation varies from 1.45 in *Terrapene carolina* (Jordaan 1938), 1.73 in *Testudo hermanni* (Uğurtaş et al. 2003),

1.80 in *P. geometricus* (Bernstein 1938) and 2.12 in *Testudo horsfieldii* (Knotkova et al. 2002). Nevertheless, both size and shape are influenced by cohort and season and it is best to interpret variation in erythrocyte morphology in such context.

3.4.2 Effects of cohort upon mature erythrocytes of *H. areolatus*

Haemoglobin plays a functional role in oxygen uptake and delivery and is present in the cytoplasm of erythrocytes (Strik et al. 2007; Quigley et al. 2014). The functional properties of haemoglobin are well adapted to meet the metabolic needs of an individual (Torsoni et al. 2002). The nuclear to cellular area ratios and pixelation values are relevant as they indicate erythrocyte maturity. Mature erythrocytes are characterised by small nuclear to cellular area ratios and have high pixelation values. The high cytoplasmic pixelation values are an indication of the haemoglobin present within the erythrocyte (Campbell 2012; Quigley et al. 2014). Small, mature, ellipsoidal erythrocytes transport oxygen most efficiently (Hartman and Lessler 1964).

Female erythrocytes of *H. areolatus* overall, were the smallest with the shortest widths and were less ellipsoidal than that of males. Similarly, Walton (2012) found that female erythrocytes were smaller compared to that of males in *Psammobates geometricus*. Pixelation values and nucleus to cellular area ratios were intermediate between males and juveniles. The morphology of female erythrocytes of *H. areolatus* suggests that they were mature, small and ellipsoidal. Consequently, erythrocytes of females were able to contribute well to the process of gaseous exchange. The latter is particularly important considering the fact that females require higher energy levels for reproduction activities such as vitellogenesis, ovulation and nesting. Furthermore, female body sizes among individuals of *H. areolatus* are largest compared to that of males and juveniles (Branch 1989). To meet the high metabolic needs of female tortoises, erythrocytes are required to transport oxygen most efficiently. The mature, small, ellipsoidal erythrocyte probably helped to facilitate the delivery of oxygen in females.

Erythrocytes of males were larger and more ellipsoidal than females. In addition, male erythrocytes were most pixelated and had the smallest nucleus to cellular area ratios. Larger ectotherms require more energy to meet metabolic needs. The fact that males had larger erythrocytes compared to females suggests that optimal, efficient oxygen delivery was compromised. However, the small body size of males compared to that of larger females, compensated for the reduction in oxygen exchange in males. While females participate in mating and nesting, males presumably only participate in

mating, subsequently, reproduction is less taxing on males (M.D. Hofmeyr, pers. comm.).

The size of erythrocytes in juveniles, on the other hand, was intermediate between males and females. Juvenile erythrocytes were broadest and least ellipsoidal, suggesting that erythrocytes were spherical. Additionally, erythrocytes were least pixelated with relatively high nucleus to cellular area ratio. The morphology of juvenile erythrocytes alludes to the presence of immature erythrocytes. Erythropoiesis in young ectotherms is not rare (Campbell 2004). Pienaar (1962) postulated that the prevalence of immature erythrocytes in juvenile reptiles might be attributed to their faster growth rates relative to that of adults. Additionally, juveniles will need to feed more to keep up with their increasing metabolic rate associated with increased growth rates (Brown et al. 2005; Mitchell et al. 2012). The small body size of juvenile tortoises is advantageous since it enables them to be more active (Wilson et al. 1999). The latter suggests that juveniles are able to feed more to compensate for their fast metabolism associated with their rapid growth rates.

3.4.3 Effects of season upon mature erythrocytes of *H. areolatus*

Reptiles regulate their body temperatures using environmental temperature to maintain a certain body temperature for specific activities (Stevenson et al. 1985; Loehr 2012; Mitchell et al. 2012). Seasonal changes in temperature and rainfall affect food availability, which influences the metabolism of ectotherms (Wood 1980; Litzgus and Hopkins 2003; Sheridan and Bickford 2011; Setlalekgomo et al. 2012). Higher temperatures are accompanied by higher metabolic rates which allows for higher activity (Litzgus and Hopkins 2003; Mitchell et al. 2012). On the other hand, Keswick (2012) reported that in *Psammobates oculifer* activities were reduced at lower winter temperatures.

Stawski et al. 2006 reported that temperature has a direct effect on blood oxygen affinity. Since the role of erythrocytes is to transport haemoglobin that carries oxygen to the tissues, the size and shape of erythrocytes are important indicators of the surface area available for gaseous exchange to meet respiratory demands (Hartman and Lessler 1964). Small, mature ellipsoidal erythrocytes transport oxygen more efficiently than mature, large and spherical erythrocytes (Hartman and Lessler 1964). One may therefore infer that small, ellipsoidal erythrocytes will be more prevalent in extremely active individuals with high metabolic rates.

The cell area of female erythrocytes were smaller than males but relatively equal to erythrocytes of juveniles. Female erythrocytes were less elongated than males but more elongated compared to juveniles during winter. Females of *H. areolatus* undergo vitellogenesis and ovulation during winter (M.D. Hofmeyr pers. comm.). Furthermore, females begin to nest from the following August, October to November (Branch 1989). Considering the taxing reproduction events endured by females during winter, the size and shape of erythrocytes probably facilitated oxygen transport and delivery.

The blood profile of males showed that erythrocytes were larger compared to that of females and juveniles during winter. Since large erythrocytes transport oxygen less efficiently than smaller ones, this alludes to males being less active amongst cohorts during winter. Similarly, in a study conducted by Walton (2012) during winter, the erythrocytes of males of *P. geometricus* were longer and broader compared to females.

Particularly during winter when temperatures are low, ectotherms are expected to have lower metabolic rates (Lagarde et al. 2002; Litzgus and Hopkins 2003; Kassab et al. 2009; Homyack et al. 2010; Loehr 2012; Setlalekgomo et al. 2012). For juvenile tortoises, foraging during winter with low metabolic rates may become challenging, as they have to maintain a certain level of activity. Growth rates of juveniles are higher in juveniles than they are adults, consequently juveniles need to feed much more (Brown et al. 2005).

Overall, among all cohorts, erythrocytes were larger during winter and autumn than in spring and summer. At Elandsberg, rainfall commenced in late autumn (Fig. 2.1), which stimulated plant growth, providing an abundance of food by winter. Literature supports the phenomenon of increased plant growth during winter due to abundant rainfall, thereby creating an increase in food availability for tortoises (Henen 1997; Joshua et al. 2010; Loehr 2012; Walton 2012). The latter implies that sufficient food was available during spring, but as precipitation events decreased, food became progressively scarcer through summer, with autumn as the driest season (Walton 2012). Additionally, the erythrocytes for all cohorts during winter were large and least elongated. Walton (2012) speculated that winter rainfall might cause haemodilution, which could explain the large erythrocytes observed among cohorts. Furthermore, nucleus to cellular area ratios for all cohorts were relatively high, while their pixelation values were lowest during winter. The latter suggests an increase in erythropoiesis, possibly in response to the abundant food and water resources available during

winter. Similar results were reported in chapter 2.4.2. The abundance of food and water resources may have elicited the erythropoietic response during winter (Walton 2012).

Similar to winter, spring is associated with abundant food resources. However, spring is accompanied by higher temperatures (Fig. 2.1), which is associated with increased metabolic activity (Mitchell et al 2012). One may expect more energy to be exerted across cohorts during spring. The energy requirements in females during spring are exceptionally high considering their larger body sizes and their reproduction (Henen 2002; Loehr et al. 2009). Reproduction activities of female tortoises include vitellogenesis, ovulation and nesting (Branch 1989; Loehr et al. 2004). Although female erythrocytes were small during spring, their nuclei widths were relatively short, and ellipsoidal, suggesting that their nuclei were oval rather than round. In addition to the nucleus being oval, the cell shape was ellipsoidal, and the high pixelation values suggest that female erythrocytes were mature during spring. The latter is significant as matured, small, ellipsoidal erythrocytes transport oxygen more efficiently than large, spherical erythrocytes (Hartman and Lessler 1964). Similar to females, *H. areolatus* males showed evidence of having a preponderance of small, mature erythrocytes in spring, yet the high nuclear to cellular ratio indicates that erythropoiesis may be continuing.

The erythrocytes of juveniles during spring appeared to be relatively immature since erythrocytes were large with high nucleus to cellular area ratio values. In addition, erythrocytes of juveniles had lower pixelation values compared to females and males, and appeared more spherical than ellipsoidal. These values are indicative of erythropoiesis at its last phase (polychromatophilic erythrocytes as described in chapter 2.3.3). The continuation of abundant food and growth rates, particularly in juveniles, could have caused the proliferation of immature erythrocytes during spring as proposed in Walton (2012). Another possibility is that erythropoiesis may have been elicited due to an increase in temperature (Wilson et al. 1999; Mitchell et al. 2012).

During summer, female erythrocytes within *H. areolatus* were intermediate compared to that of males and juveniles. Similarly, pixelation values and nuclear to cellular area ratios were intermediate compared to males and juveniles. Despite the high temperatures (Fig. 2.1) and the possible high metabolic rates, females of *H. areolatus* during summer did not indicate signs of physiological stress during the dry summer.

Possible reasons for the morphology of female erythrocytes during summer is possibly linked to the advantage of larger bodies being less vulnerable to dehydration (López-Ortiz and Lewis 2004; Moulherat et al. 2014). Furthermore, literature supports the fact that tortoises are able to feed optimally during spring and to preserve resources for the drier months. Henen (1997) reported that females of *G. agassizii* (desert tortoise) were able to store energy before winter and utilize those reserves in the following reproduction cycle.

Erythrocytes of males during summer were largest, compared to females and juveniles. One would expect that summer would bring about physiological stress; instead, erythrocytes were large, with high pixelation values and low nucleus to cellular area ratios. Branch (1989) reported that individuals of *H. areolatus* appear to have adapted well physiologically, to cope with high temperatures. In addition, to contend with high temperatures tortoises are able to change their pattern of activity, to a bimodal pattern during summer (Ramsay et al. 2002). Additionally, foraging appears less taxing on males due to their body size.

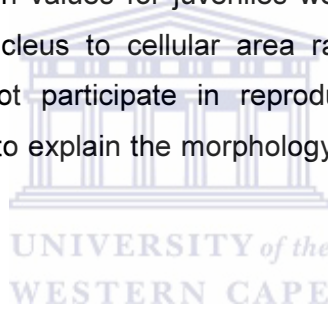
During summer, juvenile erythrocytes were relatively small with short length and widths. Pixelation values were the lowest with the highest nucleus to cellular area ratio. The latter indicates the presence of more immature rather than mature erythrocytes within the peripheral blood of juveniles. A plausible explanation for erythropoiesis in juveniles may be due to increased temperatures and limited resources. Since the metabolic rate of ectotherms increase with temperature, the metabolic rate of juveniles is probably more accelerated due to their need for increased growth rates compared to that of females and males (Pienaar 1962; Brown et al. 2005, Mitchell et al. 2012).

During autumn, the blood profile of females showed evidence of erythropoiesis since erythrocytes were the smallest, with the shortest lengths and widths suggesting that they are small and spherical. In addition, the pixelation values were the lowest and females had the highest nuclear to cellular area ratio compared to males and juveniles. The pressures of the dry season (autumn) could have elicited an erythropoietic response. The fact that food is scarce and females have to forage over longer distances may serve as another plausible explanation. Furthermore, females are larger than males and juveniles and will need to feed more as their energy

requirements are more. Furthermore, females of *H. areolatus* ovulate in autumn, which requires additional energy.

Males however, had the largest erythrocytes during autumn with the longest length and widths. Pixelation values were the highest and nucleus to cellular area ratio was intermediate between females and juveniles. Male erythrocytes therefore appeared to be large, ellipsoidal and matured. There is no evidence of physiological stress exerted upon males considering the exposure to the dry season (autumn). In principle, autumn was accompanied by high temperatures (Fig. 2.1), which is associated with high metabolic rate. However, literature supports individual ectotherms limiting their activity to slow down their metabolism (Longshore et al. 2003; Loehr et al. 2009; Keswick 2012).

The overall size of juvenile erythrocytes during autumn was intermediate between males and females. Pixelation values for juveniles were higher than in females and juveniles had the lowest nucleus to cellular area ratios compared to all cohorts. Considering juveniles do not participate in reproduction, more energy may be preserved. The latter serves to explain the morphology of juvenile erythrocytes during autumn.



3.5 CONCLUSIONS

Overall, small erythrocytes were most prevalent during higher temperatures within spring and summer, while larger erythrocytes were most prevalent during lower temperatures of the colder months. The blood profile of *H. areolatus* showed that to overcome physiological stress in warmer, drier months they relied on smaller more elongated erythrocytes. Among seasons, erythropoiesis appeared more prevalent during spring and winter. Erythropoiesis among seasons served as indicators of both physiological stress (autumn) and good nutritional status (winter and spring).

Overall, cohort effects showed that males had the largest erythrocytes. Males had the largest erythrocytes because they required the least amount of energy. Juvenile erythrocyte size was intermediate between female and males. Females however, had the greatest energy requirements to support reproduction activities such as vitellogenesis, ovulation and nesting. Furthermore, erythropoiesis overall appeared to be more prevalent in juveniles and females.

In my evaluation, I found that research regarding morphological characteristics of South African tortoises is gravely limited. I was able to link the morphology of erythrocytes to the physiology of *H. areolatus*. However, I found it particularly challenging to link the effects of season and cohort to specific behavioural and reproduction patterns. Research on the ecology and phenology of *H. areolatus* is virtually non-existent. I recommend that further ecological studies be conducted to assist scientists, conducting histological studies like these, to make conclusive deductions. Further studies regarding haematological baseline values and the effects of season and cohort on erythrocytes for tortoises are recommended. Not only is this a fascinating research topic, but rather a great demand for further studies to help scientist understand how ectotherms (particularly tortoises) respond physiologically to intrinsic and extrinsic factors.



4 LEUKOCYTE AND THROMBOCYTE HISTOLOGY

4.1 INTRODUCTION

The blood profile of reptiles include erythrocytes, thrombocytes and leukocytes (Pendle 2006; Campbell 2012). Thrombocytes have the same function as platelets do in mammals. However, thrombocytes differ in morphology and developmental pathways (Harvey 2012). The shape of thrombocytes in reptiles vary from round to oval and may appear as single cells or in aggregation (Strik et al. 2007; Nardini et al. 2013). Unlike mammalian platelets, thrombocytes are nucleated and the nucleus stains a deep dark purple (Campbell 2012). The cytoplasm of thrombocytes stains a pale blue or sometimes transparent colour, while the nucleus to cellular area ratio in thrombocytes is relatively high (Strik et al. 2007; Campbell 2012). Mammalian platelets, on the other hand, are small round-oval cytoplasmic fragments of megakaryocytes (Mader 1997; Stacy et al. 2011; Harvey 2012). Platelets do not have nuclei and their cytoplasm is light blue with reddish-purple granules (Harvey 2012).

Platelets derive from haemopoietic stem cells, which give rise to progenitor cells (Russel 2010; Harvey 2012). Progenitor cells differentiate into promegakaryocytes, which develop into megakaryocytes, and ultimately produce platelets (Mader 1997; Russel 2010). The developmental pathway for thrombocytes starts with a haematopoietic stem cells that emerges into two different cell lineages, namely the myeloid and lymphoid cell lineage (Sypek and Borysenko 1988; McGeady et al. 2006; Brody 2012). The myeloid progenitor is pluripotent and differentiates into more specialised progenitor cells, namely the thromboblats, rubriblasts, monoblasts and myeloblasts (Sypek and Borysenko 1988; Brody 2012). Through the process of thrombopoiesis, thromboblats produce immature thrombocytes (Campbell 2012).

Leukocytes of reptiles have varying origins, for instance, monoblasts produce monocytes. Monoblasts are derived from the pluripotent myeloid progenitor (Sypek and Borysenko 1988; Brody 2012; Campbell 2012). Lymphocytes are derived from the lymphoid lineage (Sypek and Borysenko 1988; Brody 2012; Campbell 2012). Blood-borne stem cells that lodge in the thymus produce the first lymphocytes in reptiles (Campbell 2012). Two precursor cell lineages respectively give rise to the B and T lymphocytes, which cannot be distinguished morphologically. The B lymphocytes differentiate further into plasma cells (Theml et al. 2004; Campbell 2012).

Furthermore, leukocytes in the peripheral blood of reptiles include granulocytes and agranulocytes. Granulocytes include heterophils, eosinophils and basophils, while agranulocytes include lymphocytes, plasma cells, monocytes and azurophils (Stacy et al. 2011; Zhang et al. 2011). In mammals neutrophils have the same function as heterophils do in reptiles (Work et al. 1998; Knotkova et al. 2002; Strik et al. 2007). Heterophils as in mammals, are responsible for fighting off infections and inflammation (Campbell 1996; Stacy et al. 2011). Toxic heterophilia indicate disease or stress (Hawkey and Dennett 1989; Strik et al. 2007; Campbell 2012). In chelonians eosinophils help to combat parasitic and bacterial infections (Strik et al. 2007; Bell and Gregory 2014). Basophils defend the body against chronic and long-term illness. Additionally, basophils help to rid the body of haemoparasites and inflammation in mammals and reptiles (Hawkey and Dennett 1989; Strik et al. 2007) Lymphocytes assist in wound healing and curing infections and inflammatory diseases (Nardini et al. 2013; Campbell 2012). Plasma cells assist with inflammation and helps to fight off infections (Nardini et al. 2013). Monocytes in both mammals and reptiles fight off foreign and bacterial substances (Strik et al. 2007; Davis et al. 2008). Azurophils however, are not present in mammals, are rarely encountered in chelonians, but are prevalent in the blood of snakes, squamates and crocodiles (Lisičić et al. 2003; Strik et al. 2007; Stacy et al. 2011). Additionally, azurophils aid in inflammatory reactions (Strik et al. 2007; Nardini et al. 2013)

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Leukocyte composition differ among species, cohorts, season and due to defenses against pathogens (Duguy 1970; Davis et al. 2008; Mendoza-Rangel et al. 2009; Lisičić et al. 2013; Arizza et al. 2014; Bell and Gregory 2014). Among reptilian species, azurophils and lymphocytes were most abundant in *Vipera ammodytes*, whereas lymphocytes, heterophils and eosinophils were most prevalent in *Chelonia mydas* (Oliveira-Júnior et al. 2009; Lisičić et al. 2013). Stacy et al. (2011) reported that individuals of *Caretta caretta* usually have higher lymphocyte than heterophil counts. Furthermore, Zhang et al. (2011) reported that basophils in *Mauremys mutica* were the most common leukocyte type while they were rare in turtle species such as *C. caretta* and *C. mydas*.

Leukocyte differences among reptilian cohorts are evident (Tripathi and Singh 2014). In *V. ammodytes* males appeared to have significantly higher eosinophil counts compared to that of females (Lisičić et al. 2003) whereas among females of *Vipera berus* lymphocytes were significantly higher compared to that of males (Duguy 1970). Juveniles of *Vipera aspis* tend to have higher basophils counts in their peripheral

blood compared to those of adults (Duguy 1970). Amongst *Mauremys caspica* females, lymphocytes were more prevalent compared to that of males during spring and summer, reportedly due to higher activity during those seasons (Muñoz et al. 2004; Bell and Gregory 2014).

Eosinophils, monocytes and basophils are least affected by seasonal variation (Duguy 1970; Otis 1973; Sacchi et al. 2007). Particularly among snakes, eosinophils are known to be rare, with low eosinophil counts during summer (Bell and Gregory 2014). Generally, during hibernation of reptiles, heterophil counts tend to be low, followed by an exponential increase during summer (Duguy 1970). Among individuals of *Chrysemys picta*, heterophil to lymphocyte ratios increased during hibernation (Schwanz et al. 2011). Furthermore, *Emys orbicularis*, *Anguis fragilis* and *Natrix maura* presented high lymphocyte values during summer and low lymphocyte values during hibernation (Duguy 1970).

Differential and total white blood cell counts vary according to their role in immunity (Chen et al. 2007; Mendoza-Rangel et al. 2007; Davis et al. 2008; Lisičić et al. 2013). For instance, the percentage of eosinophils usually increases with an increase in haemoparasites (Mendoza-Rangel et al. 2009). In addition the heterophil to lymphocyte ratio is a measure of stress (Chen et al. 2007; Schwanz et al. 2011), suggesting that leukocytes are key indicators of disease and physiological stress (Davis et al. 2008; Paul et al 2008; Zhang et al. 2011). Furthermore, stress hormones reportedly increase the number of heterophils (=neutrophils) in mammals and amphibians, but decrease the number of lymphocytes among all vertebrate species (Bell and Gregory 2014).

Since reptiles are affected by ambient temperatures, physiological changes are evident in their blood profiles (Paul et al. 2008). I employed haematology to provide baseline values, which in future can help distinguish normal fluctuations from those that reflect disease. and physiological stress. The objectives of this chapter were to (1) provide detailed descriptions of the histology of thrombocytes and leukocytes of *H. areolatus*, (2) assess if the leukocyte and thrombocyte profiles differ among cohorts, and (3) evaluate how seasonal changes in environmental conditions and physiological status of cohorts influence thrombocyte and leukocyte profiles.

4.2 MATERIALS AND METHODS

See Chapter 2 for a description of field procedures, as well as the staining of blood smears and basic procedures of histological evaluations of cell types (thrombocytes and leukocytes in this instance).

4.2.1 Histological evaluations and differential white cell counts

I first familiarised myself with the histological appearance of all cell types in the blood smears to be able to identify thrombocytes and the different leukocyte types with certainty. Subsequently, I photographed representative cells at 1000x magnification under immersion oil. I photographed 30 cells of each common leukocyte type as well as 30 thrombocytes for measurement. Since basophils and monocytes had low frequencies, I photographed and measured only ten cells of each. Cells that appeared even less frequently than basophils and monocytes were plasma cells and azurophils. Only four plasma cells and two azurophils were measured.

All micrographs were saved as jpeg files at 2048 x 1536 pixels and were kept as a permanent record. I used the digital images to perform cell and nuclear measurements with Nikon NIS Elements imaging software in a similar manner as described for erythrocytes in chapters 2 and 3. Broadly, this entailed that I used the contrast and white saturation functions to reduce staining artifacts and intensify nuclear and cellular boundaries before activating automated measurements of cell and nuclear dimensions. The contrast and white saturation functions were not sufficient to accurately distinguish the cell and nuclear parameters from darkly stained leukocytes such as plasma cells and azurophils. Consequently due to the lack of accurate measurements for nuclear parameters, no nuclear measurements were recorded for plasma cells and only one nuclear measurement for azurophil was recorded. Furthermore, it was not possible to measure the nuclei of basophils because the dark cytoplasmic granules overlaid the nucleus to such an extent that it was impossible to obtain a clear nuclear outline. Similarly, it was difficult to measure nuclear dimensions of some monocytes because of the irregular nuclear shape. After measurements were completed, data were exported to Windows Excel 2007 (MS Office) and collated into one spreadsheet for analysis.

I used the meandering technique to perform differential white blood cell counts for blood smears of all individuals. I searched each slide from side to side and noted the type of leukocyte encountered until I have counted 100 leukocytes. Whilst doing the

differential white cell count, I recorded each thrombocyte encountered so that the number of thrombocytes could ultimately be expressed relative to 100 leukocytes. Subsequently, the data were transcribed to Microsoft Excel for further analysis.

4.2.2 Data and statistical analysis

I used SigmaStat (SPSS Inc., Chicago, U.S.A. version 2.03). My objectives with the analyses were to (1) obtain descriptive statistics for cell and nuclear measurements of leukocyte types and thrombocytes, (2) evaluate differences in measurements among cell types (3) assess if differential white cell counts differ among cohort and season and (4) establish an index of thrombocyte abundance and assess if the index differed among cohorts and seasons. I used data transformation to normalise data in order to use multiway ANOVAs but when two-way ANOVAs were not possible, one-way ANOVAs were performed using *F* statistic for parametric data and Kruskal-Wallis ANOVAs on ranks (*H* statistic) for non-parametric data. For post hoc comparisons, Student-Newman-Keuls were used for parametric one-way ANOVAs, whereas Dunn's post hoc comparisons were used for Kruskal-Wallis ANOVAs. To analyse the leukocyte and thrombocyte counts I performed square root transformations in SigmaStat. Square root transformations were sufficient for most parameters to meet the parametric requirements, although certain tests failed normality and equal variance.

4.3 RESULTS

4.3.1 Leukocyte and thrombocyte morphology

I encountered seven types of leukocytes as well as thrombocytes in the peripheral blood of *H. areolatus*. The leukocytes consisted of three types of granulocytes, the heterophils, eosinophils and basophils, and four types of agranulocytes, which included lymphocytes and their modified form, the plasma cell, as well as monocytes and their modified form, the azurophils.

Heterophils in the circulating blood of *H. areolatus* were large cells, ranging from 81.31 to 222.31 μm^2 (Table 4.1). The shape of heterophils was relatively oval (elongation = 1.3 ± 0.2) and the cell contained transparent cytoplasm, filled with pink to orange or even red, spindle-shaped cytoplasmic granules, giving an overall pixelation of 201.5 ± 30.7 (Fig. 4.1a,b). The nucleus was either single or bi-lobed (Fig. 4.1a,b), and nuclear area ranged from 21.95 to 66.55 μm^2 . The nucleus was located eccentrically and its shape was mostly oval (elongation = 1.4 ± 0.2). The nucleus

stained darkish blue (pixelation = 138.2 ± 37.9). The nucleus often contained one or more nucleoli and the chromatin network appeared coarse and scattered. Additionally, heterophils are known to ingest particles within the cytoplasm (Fig. 4.1c). I also observed toxic heterophils (Fig. 4.1d). The main difference between normal and toxic heterophils is the shape and colour of granules. Toxic heterophils have bright red to orange round coarse granules.

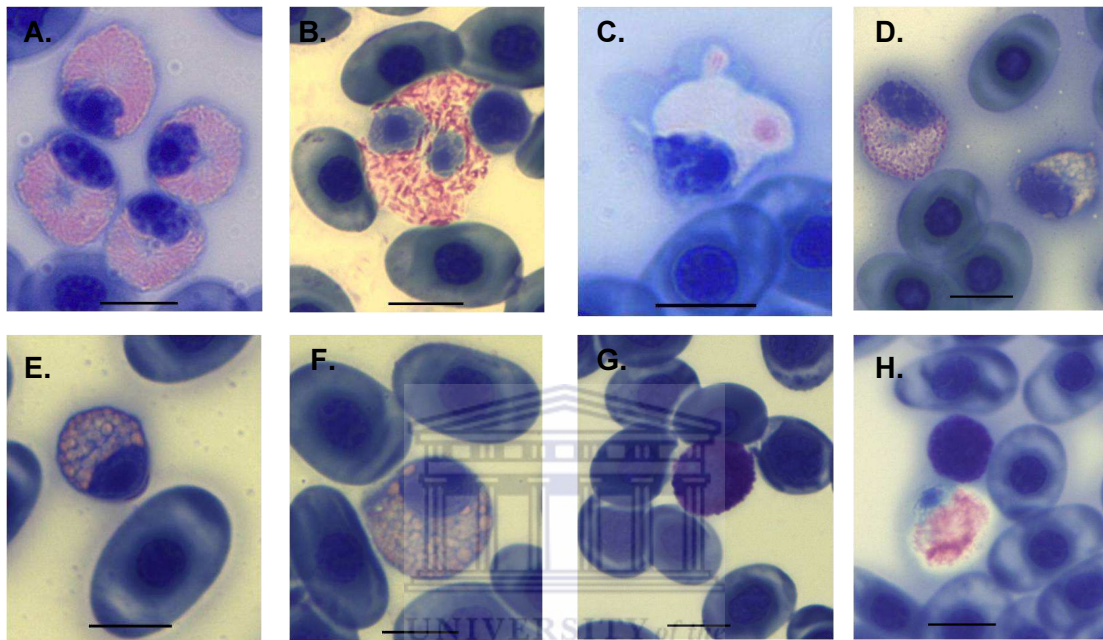


Figure 4.1 Granulocytic leukocytes in the peripheral blood of *Homopus areolatus*. (a) Four uni-lobed heterophils, (b) a bi-lobed heterophil with spindle-shaped granules, (c) a reactive heterophil with a pseudopod and cytoplasmic inclusions and (d) a toxic heterophil on the left with a heterophil on the right. (e) Eosinophils have round granules and an off-centric nucleus with a single nucleolus, or (f) multiple nucleoli. (g,h) Basophils appear as round cells with dark-staining granules obscuring the nucleus. The scale bar represents 10 μm at 1000 x magnification.

Eosinophil cells were relatively large and cell area ranged from 60.9 to 184.9 μm^2 (Table 4.1). The shape of the cell was spherical rather than oval, with a mean elongation of 1.2 ± 0.1 . The cytoplasm was light basophilic and contained round, pinkish-red granules giving the cell an overall pixelation of 176.5 ± 24.5 (Fig. 4.1e,f). The eccentric nucleus of eosinophils was either single or bi-lobed and ranged from 21.1 to 60.9 μm^2 (Fig. 4.1e,f). The shape of the nucleus was relatively round rather than oval (elongation = 1.2 ± 2.2). The nucleus of eosinophils stained dark blue

(pixelation = 129.0 ± 24.6). The nucleus contained one or more nucleoli and the chromatin was coarse and dispersed.

Table 4.1 Cellular and nuclear area, length and width, as well as the ratio of nuclear to cellular (N/C) areas of leukocytes and thrombocytes in *Homopus areolatus*. Nuclei of basophils and plasma cells could not be measured since the dark cytoplasmic granules or overall dark staining obscured the nucleus. Similarly, it was only possible to measure one nucleus of the two azurophils measured. Data are presented as means and standard deviations of 30 cells of each cell type except for basophils ($n=10$), monocytes ($n=10$), plasma cells ($n=4$) and azurophils ($n=2$).

Cell type	Structure	Area (μm^2)	Length (μm)	Width (μm)	N/C ratio
Heterophil	Cell	152.8 ± 37.1	16.2 ± 3.6	9.5 ± 1.8	
	Nucleus	40.7 ± 9.1	8.7 ± 1.7	4.7 ± 0.6	0.41 ± 0.09
Eosinophil	Cell	113.4 ± 33.0	13.3 ± 2.0	8.4 ± 1.4	
	Nucleus	37.80 ± 11.4	8.6 ± 1.5	4.3 ± 0.7	0.34 ± 0.05
Basophil	Cell	100.1 ± 29.8	12.1 ± 2.0	8.1 ± 1.3	
Lymphocyte	Cell	99.0 ± 31.3	12.0 ± 2.3	8.0 ± 1.1	
	Nucleus	75.3 ± 24.8	10.5 ± 2.0	7.0 ± 1.2	0.76 ± 0.05
Monocyte	Cell	110.9 ± 31.5	12.9 ± 1.6	8.4 ± 1.4	
	Nucleus	70.4 ± 19.9	12.3 ± 2.9	5.7 ± 0.8	0.67 ± 0.07
Plasma cell	Cell	143.1 ± 69.5	14.0 ± 3.4	9.7 ± 2.5	
Azurophil	Cell	162.9 ± 80.2	15.0 ± 3.6	10.6 ± 2.8	
	Nucleus	77.8	10.4	7.5	0.73
Thrombocyte	Cell	46.7 ± 10.6	8.5 ± 1.8	5.4 ± 0.6	
	Nucleus	33.4 ± 8.5	7.7 ± 1.7	4.4 ± 0.8	0.72 ± 0.09

Cell areas of basophils within *H. areolatus* ranged between 70.7 to $155.4 \mu\text{m}^2$ (Table 4.1). The cells were round (elongation = 1.1 ± 0.1) with clear cytoplasm containing many large, round dark-purple granules (Fig. 4.1g,h). Basophils had the darkest appearance (pixelation = 74.1 ± 35.4) of all the leukocytes. The outline of the cell often looked scalloped due to the large granules near the surface. Most often, it was not possible to discern characteristics of the nucleus because it was obscured by the large cytoplasmic granules.

Homopus areolatus had small and large lymphocytes (Fig. 4.2 a-c). Cell area ranged between 57.2 and 173.3 μm^2 and the shape was distinctively spherical (elongation = 1.1 ± 0.1). The cytoplasm stained light blue and was visible as a thin rim around the large nucleus. The cells stained relatively dark with a pixelation of 144.2 ± 24.5 (Fig. 4.2a-c). The lymphocyte nuclear area of *H. areolatus* ranged between 40.9 to 140.7 μm^2 . The nucleus was centrally located with an almost spherical shape (elongation = 1.1 ± 0.1). The nucleus stained darkish blue (pixelation = 136.9 ± 25.0) and contained a relatively coarse chromatin network. The peripheral blood occasionally contained lymphocytes with cytoplasmic protrusions (Fig. 4.2c).

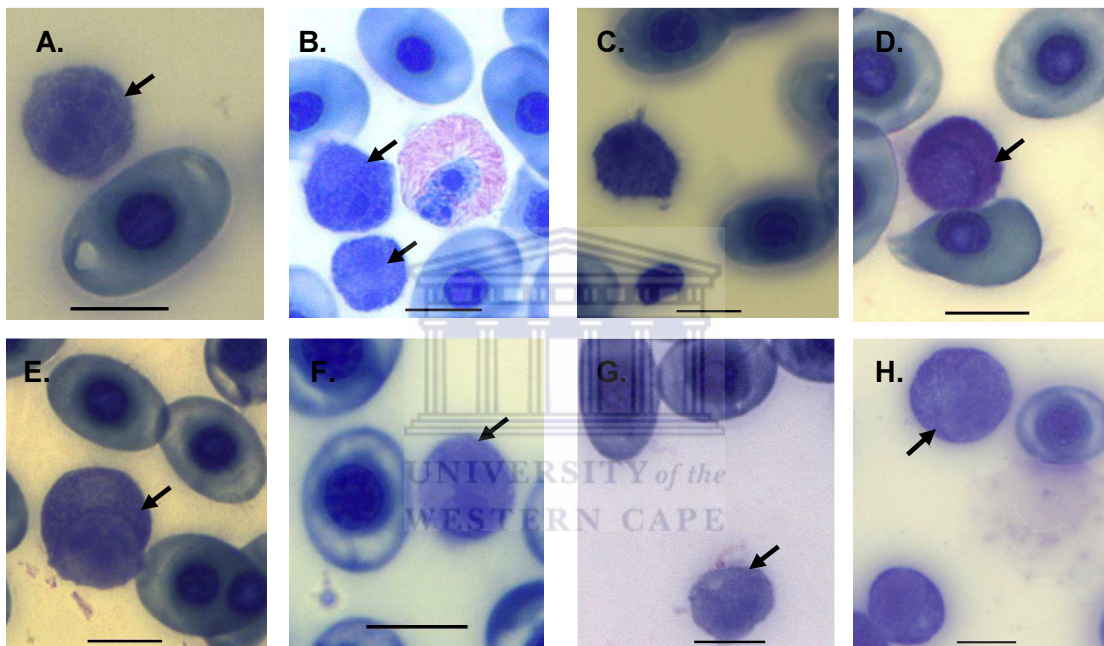


Figure 4.2 Agranulocytic leukocytes in the peripheral blood of *Homopus areolatus*. (a) A lymphocyte showing a large nucleus and thin rim of cytoplasm, (b) two lymphocytes of varying size, (c) a lymphocyte with protrusions, (d,e) plasma cell (f,g) monocytes with typical indented nucleus and basophilic granular cytoplasm and (h) an azurophil. The scale bar represents 10 μm at 1000 x magnification.

Plasma cells were present but were few in number with size ranging from 77.4 to 219.6 μm^2 (Table 4.1). The shape of plasma cells varied from round to sometimes oval with a mean elongation of 1.1 ± 0.0 (Fig. 4.2d-e). The cytoplasm of plasma cells stained dark blue (pixelation = 90.9 ± 43.9) and contained intensely basophilic cytoplasm with a perinuclear halo (Fig. 4.2 d-e). The size of the nucleus varied from cell to cell and the shape varied from round to oval and was eccentrically located.

When visible, the nucleus stained dark blue and the chromatin network was coarsely clumped.

The cell areas of monocytes in *H. areolatus* were large, although I also encountered smaller cells, ranging from 77.9 to 164.3 μm^2 (Table 4.1). The shape of monocytes was round to oval (elongation = 1.2 ± 0.1) and the agranular cytoplasm stained light blue, giving a pixelation of 155.9 ± 20.7 (Fig. 4.2f-g). The nucleus often resembled the shape of a bean or “kidney-shaped” (Fig. 4.2g). The size of nucleus ranged between 48.4 to 100.0 μm^2 and the nucleus was often slightly eccentrically located. The nucleus stained blue to a light purple-greyish colour (pixelation = 159.6 ± 14.8). The chromatin network was smooth and condensed. Monocytes were present but uncommon in *H. areolatus*.

I only encountered a few azurophils, which were large (106.3 to 219.6 μm^2), round cells. The entire cell stained purplish blue with little distinction between the nucleus and cytoplasm (Fig. 4.2h). The cytoplasm contained round blue-purple granules giving the cell an overall pixelation of 165.7 ± 15.1 . The nucleus was large, round and slightly irregular in outline and was eccentrically located (Fig. 4.2h).

Thrombocytes of *H. areolatus* varied in size (29.8 to 70.8 μm^2) but were typically smaller than other leukocytes (Table 4.1) and the shape varied from round to oval (elongation = 1.2 ± 0.2). The agranular cytoplasm of thrombocytes stained light blue (Fig 4.3a-c) but because the thrombocytes contained little cytoplasm and large, dark nuclei, pixelation for the cells was relatively low (138.6 ± 22.5). The nucleus varied from round to oval (elongation = 1.2 ± 0.2). In addition, the nucleus was centrally positioned, but occasionally I observed it to be eccentrically positioned (Fig. 4.3 b). The nuclear contents stained dark blue to dark purple (pixelation = 128.5 ± 22.5) and contained a smooth, condensed chromatin network (Fig. 4.3c).

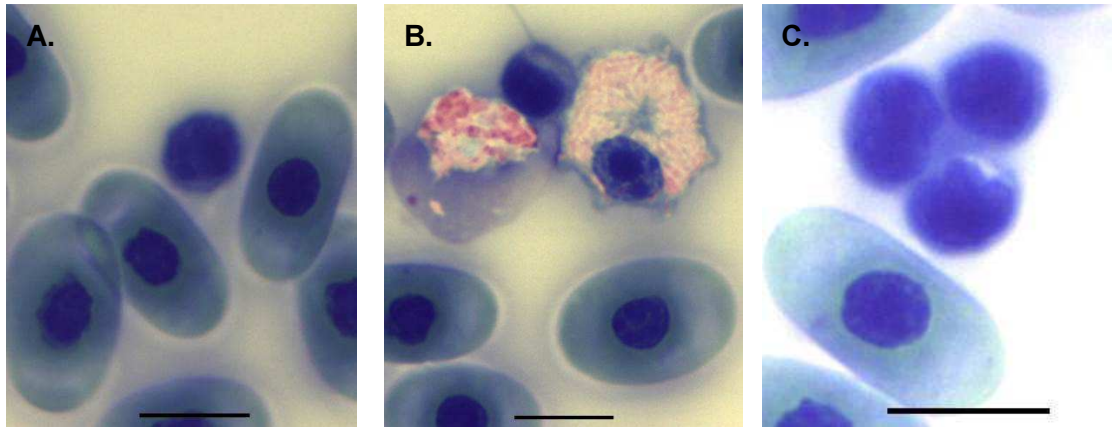


Figure 4.3 Thrombocytes in the peripheral blood of *Homopus areolatus*. (a) A thrombocyte showing a round, large basophilic nucleus with scant rim of clear cytoplasm, (b) thrombocyte with cytoplasmic protrusions, and (c) three thrombocytes in aggregation. The scale bar represents 10 μm at 1000 x magnification.

4.3.2 Cell and nuclear dimensions of leukocytes and thrombocytes

The cell area, cell length and cell width of all leukocytes were larger than that of thrombocytes ($F_{7,137} > 23.27$, $P < 0.0001$; Table 4.1). In addition, heterophil cell length was greater than that of lymphocytes, basophils, monocytes and eosinophils. Cell pixelation values were lowest for basophils and plasma cells. Additionally, pixelation of heterophils and eosinophils was greater than in lymphocytes and thrombocytes with that of heterophils also greater than for monocytes ($F_{7,137} = 34.52$, $P < 0.0001$). Although the test for differences among cell elongation was significant ($H_7 = 31.51$, $P < 0.0001$), there were no post hoc differences.

Basophils, plasma cells and azurophils were excluded from nuclear comparisons because there were no or too few measurements. Nuclear area, length and width differed among remaining leukocytes and the thrombocytes ($F_{4,120} > 14.89$; $P < 0.0001$). Nuclear area, length and width of monocytes and lymphocytes were greater than for the remaining cell types. In addition, heterophils had greater nuclear areas than thrombocytes, heterophils and eosinophils had greater nuclear lengths than thrombocytes had, whereas lymphocytes had wider nuclei than monocytes had. Nuclear pixelation did not differ among cells ($P = 0.16$) but elongation did ($H_4 = 54.65$, $P < 0.0001$). Nuclei of eosinophils, monocytes and heterophils were more elongated for than for lymphocytes, and greater for eosinophils than for thrombocytes.

The nucleus to cellular area ratio for thrombocytes and leukocytes (excluding plasma cells, azurophils and basophils) indicated that ratios for lymphocytes and thrombocyte

were higher than for eosinophils and heterophils and that monocytes also had a higher nuclear to cellular ration than heterophils had ($H_4 = 97.01$, $P < 0.0001$).

4.3.3 Differential white cell counts and effects of season and cohort

Season and cohort comparisons are based on differential white cell counts, where the contribution of each leukocyte cell type to the total leukocyte count may differ among cohorts and among seasons. In order to simplify reporting of my results, I will use symbols (smaller than, <; greater than, >; equal, =) and abbreviations for cohorts (male, M; female, F; juvenile, J), seasons (spring, Sp; summer, Su; autumn, Au; winter, Wi) and cell types (heterophil, H; eosinophils, E; basophils, B; lymphocytes, L; monocytes, M, azurophil, A, and plasma cell, PC) to summarise the outcome of comparisons among cohorts and seasons.

When I combined differential white cell counts of all seasons and cohorts for *H. areolatus*, the frequency of leukocyte types differed significantly ($H_6 = 480.1$, $P < 0.0001$). Overall, the pattern for leukocyte prevalence indicated that $H=L>E=B>PC=M=A$.

Table 4.2 Differential white cell counts of *Homopus areolatus* for cohorts and seasons combined. Results are presented as means with standard deviations (SD) as well as medians with 25 and 75% percentiles.

Cell type	Mean \pm SD	Medians (25%, 75%)
Heterophil	52.99 \pm 15.79	51.5 (43.0, 75.0)
Lymphocyte	34.16 \pm 15.55	33.0 (23.0, 45.0)
Eosinophil	8.00 \pm 7.09	7.0 (3.0, 11.0)
Basophil	3.71 \pm 2.89	3.0 (2.0, 5.0)
Plasma cell	0.73 \pm 1.42	0.0 (0.0, 1.0)
Monocyte	0.37 \pm 0.73	0.0 (0.0, 0.0)
Azurophil	0.08 \pm 0.28	0.0 (0.0, 0.0)

It was not possible to combine all factors (leukocyte type, season and cohort) in one analysis; consequently, I used two-way or one-way ANOVAs to evaluate the different factors. Furthermore, I did not include monocytes and azurophils in the analyses because their frequencies were too low. I first report the effects of season and type within each cohort. Within females, the pattern of leukocyte frequency indicated $H>L>E>B>PC$ ($F_{4,180} = 272.7$, $P < 0.0001$) but season did not change the pattern ($P =$

0.86; Fig. 4.4). Similarly, season had no effect on leukocyte pattern of either males or juveniles ($P > 0.70$). The results for leukocyte prevalence within males showed that $H>L>E>B>PC$ ($F_{4,120} = 183.5$, $P < 0.0001$). The leukocyte prevalence pattern for juveniles were different compared to females and males. Within juveniles leukocyte prevalence showed that $H=L>B=E>PC$ ($F_{4,50} = 84.3$, $P < 0.0001$ Fig. 4.4).

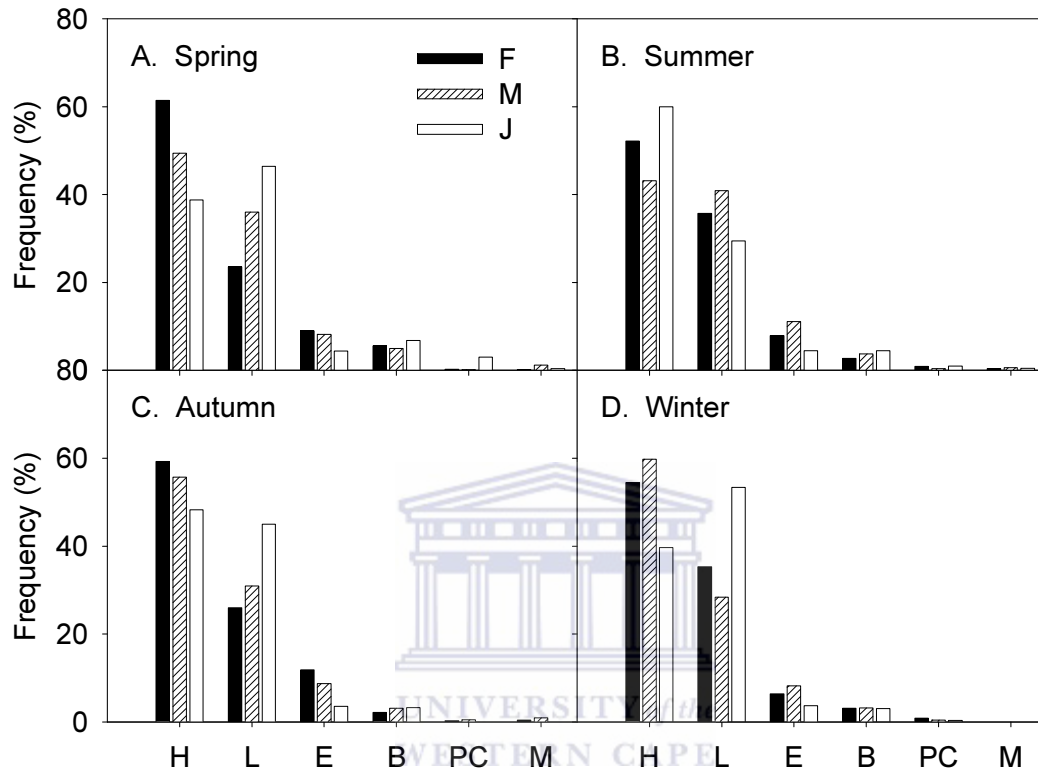


Figure 4.4 Leukocyte prevalence within *Homopus areolatus* among cohorts (female, male, juvenile) and all seasons (spring, summer, autumn, winter). Data presented as percentages.

When evaluating the effects of leukocyte type and cohort within each season, I found no effect of cohort ($P > 0.84$) but leukocyte frequency differed within each season. The results for summer showed that $H>L>E>B>PC$ ($F_{4,95} = 139.1$, $P < 0.0001$; Fig. 4.4). The pattern of leukocyte frequency for spring showed that $H>L>E=B>PC$ ($F_{4,90} = 121.7$, $P < 0.0001$). Spring also showed an interaction between cohort and leukocyte type ($F_{8,90} = 4.14$, $P = 0.0003$). Overall and for females, the leukocyte frequency pattern showed that $H>L>E=B>PC$. The frequency pattern for males showed that $H=L>E=B>PC$ and for juveniles $L=H>B=E=PC$. During autumn results changed such that $H>L>E>B>PC$ ($F_{4,95} = 116.2$, $P < 0.0001$). For winter the pattern showed that $H>L>E=B>PC$ ($F_{4,80} = 124.4$, $P < 0.0001$); Fig. 4.4).

When considering the effect of season and cohort on the frequency of leukocyte types, I found that cohort had a significant effect only on eosinophils, lymphocytes and monocytes. Eosinophils were more prevalent in males than they were in juveniles ($H_2 = 9.02$, $P = 0.011$; Fig. 4.4). Lymphocytes were more prevalent in juveniles than they were in females ($F_{2,70} = 4.07$, $P = 0.021$). Although cohort had a significant effect on monocytes ($H_2 = 8.21$, $P = 0.017$), there were no post hoc differences but there was a tendency for males to have higher values than females and juveniles. In this study, most leukocyte types were unaffected by seasonal changes ($P > 0.271$), but not basophils ($F_{3,70} = 3.98$, $P = 0.011$) and monocytes ($H_3 = 8.72$, $P = 0.0333$). Monocytes had no significant post hoc effects but tended to be lowest in winter when I detected no monocytes in any cohort member. Basophils were most prevalent in spring, with no difference among the other seasons (Fig. 4.4).

4.3.4 Thrombocyte abundance and effects of season and cohort

Thrombocyte numbers for all seasons and cohorts combined averaged 106.6 ± 18.5 cells per 100 leukocytes. When testing for the effects of season and cohort on thrombocyte frequencies, the two-way ANOVA showed an effect of season ($F_{3,70} = 15.69$, $P < 0.0001$), cohort ($F_{2,70} = 8.57$, $P = 0.0005$) and an interaction between season and cohort ($F_{6,70} = 2.72$, $P = 0.020$). Overall, thrombocyte frequency per 100 leukocytes was higher in females (111.0 ± 20.7) and males (106.0 ± 13.6) than in juveniles (95.3 ± 16.4). The frequency for thrombocytes decreased from winter in the sequence: Wi>Au>Su=Sp (Table 4.3). There were no cohort differences in autumn, spring and summer, but in winter F>M=J. There were also no differences among seasons within males or juveniles, but in females, values were highest in winter, with the number of thrombocytes also higher in autumn than in spring (Table 4.3).

Table 4.3 Thrombocyte counts for all cohorts (females, males, juveniles) among all seasons (spring, summer, autumn, winter) for *Homopus areolatus*. Data are presented as means and standard deviations.

	Spring	Summer	Autumn	Winter
Female	91.5 ± 7.3	102.2 ± 7.6	112.9 ± 9.7	136.6 ± 16.1
Male	100.0 ± 8.5	100.9 ± 11.0	107.8 ± 15.2	116.6 ± 14.1
Juvenile	87.0 ± 9.5	80.0 ± 21.2	107.3 ± 16.5	103.3 ± 11.9

4.4 DISCUSSION

4.4.1 Leukocyte prevalence and histology

The number of haematological studies of chelonians have become immensely important in recent years and literature on this topic has increased (Stacy et al. 2011; Zhang et al. 2011). Evaluation of leukocytes, more specifically heterophils and lymphocytes may be used to measure the level of stress in reptiles (Chen et al. 2007; Davis et al. 2008; Schwanz et al. 2011). Furthermore, high heterophil and low lymphocyte counts were reported in turtles associated with inflammation and infection (Adamovicz et al. 2015). Therefore, utilising heterophil to lymphocyte ratio is a good method of measuring stress levels in reptiles (Chen et al. 2007; Schwanz et al. 2011).

Among healthy chelonians, heterophils appear to be the most abundant leukocyte (Stacy et al. 2011), while eosinophils are reportedly less common (Zhang et al. 2011). However, in turtle species such as *Mauremys leprosa* and *Chelonia mydas* eosinophils were most abundant (Zhang et al. 2011). In *Agrionemys horsfieldi* (Davis et al. 2008) the frequency pattern of leukocytes was L>H>M>B>E. The pattern for the mean differential white blood cell count for *H. areolatus* (Table 4.2) indicated that H>L>E>B>P>M>A but the overall pattern was H=L>E=B>PC=M=A. Literature reports variation in leukocyte profiles due to differences among species (Duguy 1970; Strik et al. 2007; Zhang et al. 2011; Campbell 2012), which could serve as a plausible explanation for the differences seen in *A. horsfieldi* and *H. areolatus*.

In a sympatric species *Psammobates geometricus* the pattern for cell area was M>H>PC>E>A>B>L (Walton 2012). The pattern for cell area in *H. areolatus* on the other hand, showed that A>H>PC>E>M>B>L. Compared to all other leukocytes, lymphocytes appeared to have the smallest cell areas in both *H. areolatus* and *P. geometricus*. Similarly in both *H. areolatus* and *P. geometricus* plasma cells appeared to have larger cell areas than eosinophils. Differences in leukocyte profiles is attributed to due to age, season and cohort (Duguy 1970; Pienaar 1962; Cartledge et al. 2005; Strik et al. 2007, Campbell 2012).

4.4.2 Effects of cohort and season on leukocytes

In *H. areolatus* overall among cohorts, heterophils and lymphocytes were most frequent with eosinophils as the third most prevalent leukocyte. Similarly in *P. geometricus* the leukocyte frequency pattern for cohorts overall showed that H>L>E.

Females of *Terrapene carolina carolina* had higher eosinophil and basophil counts compared to that of males of the same species (Adamovicz et al. 2015). In juvenile *Crocodylus palustris* and adult *Crocodylus siamensis* heterophil counts appeared to be the most abundant leukocyte (Stacy et al. 2011).

Lymphocytes within *H. areolatus* were more abundant in juveniles than in females, which could possibly be related to rapid growth rates compared to that of adults, as proposed by Pienaar (1962). Interestingly heterophils were required more than lymphocytes in both females and males. Perhaps the high heterophil count in females was related to stress elicited by reproductive activity. Inferences for the prevalence of both heterophils and lymphocytes can be explained by a particular need to defend the body against infection, inflammation and assist in wound healing. Toxic heterophils are associated with stress and disease (Strik et al. 2007), and the fact that toxic heterophils were extremely low in frequency indicated that perhaps individuals of *H. areolatus* did not suffer from any severe stress or diseases.

Leukocyte profiles may be influenced by seasonal temperature and rainfall (Davis et al. 2008). Since food and water resources were limited during summer and autumn, the leukocyte profile during autumn is attributed to stress associated limited food availability. The seasonal frequency pattern for *H. areolatus* during summer and autumn was H>L>E>B>PC. Similarly, heterophil and lymphocyte counts in *Psammobates geometricus* were high during summer and autumn. Furthermore, heterophil and lymphocyte counts were equal in males and juveniles during spring. A plausible explanation for the latter is that physiological stress was alleviated in males and juveniles, due to less involvement in reproduction activities compared to that of females. The leukocyte profile of *H. areolatus*, during winter indicated H>L>E=B>PC, which alludes to an increased immune response attributed to seasonal fluxes (Strik et al. 2007) associated with colder months. Since eosinophils are primarily responsible for fighting off infections (Hawkey and Dennet 1989; Armando and Rovira 2010). The fact that the overall eosinophil count is low suggests that the health of *H. areolatus* was not compromised by infection. To further support the overall health status of *H. areolatus*, basophils primarily responsible for chronic illnesses and monocytes responsible warding off foreign substances also were also low in frequency within the blood profile of *H. areolatus*.

4.4.3 Thrombocyte histology and frequencies

The cell area of thrombocytes in *H. areolatus* was in accordance with thrombocyte dimensions described in *P. geometricus* (Walton 2012). Arikan and Çiçek (2014) reported thrombocyte dimensions for Testudines overall; total length and width were 13.68 ± 0.35 and $6.27 \pm 0.25 \mu\text{m}$, respectively. The length of thrombocytes ($8.5 \pm 1.8 \mu\text{m}$) in *H. areolatus* was shorter compared to the data described in Arikan and Çiçek (2014).

Thrombocytes in *H. areolatus* during spring were highest in males and least frequent in juveniles. The high thrombocyte count in males was probably elicited by a high requirement for homeostatic regulation. Similarly in *P. geometricus* thrombocyte counts were highest in males and lowest in juveniles (Walton 2012). Thrombocytes phagocytose senescent erythrocytes and leukocytes, bacteria and tissue debris (Frye 1991; Strik et al. 2007). During autumn, considering the low nutritional state related to limited rainfall, bacteria and tissue debris were most probably high, emphasizing the need for thrombocytes particularly during autumn.

This study provided a baseline haematological reference for *H. areolatus*. These reference values were comparable to the literature. The overall leukocyte and thrombocyte profile differed among season and cohort. This study may be useful in future comparative studies on South African tortoises in particular.

4.5 CONCLUSIONS

The morphology and dimensions of leukocytes and thrombocytes described in *H. areolatus* is in accordance with the literature (Strik et al. 2007, Zhang et al. 2011; Stacy et al. 2011; Campbell 2012; Walton 2012; Javanbakt et al. 2013). Overall, considering that eosinophils and basophils, associated with haemoparasites and chronic or long-term illness, were low in frequency, provides evidence to suggest that *H. areolatus* had a good clinical, healthy status. Heterophil and lymphocyte counts were most prevalent, but were in accordance with other healthy reptilian studies (Stacy et al. 2011; Walton 2012). Furthermore differences between heterophil and lymphocyte counts were evident among season and cohort.

Literature (Duguay 1970; Chen et al. 2007; Davis et al. 2008; Schwanz et al. 2011; Zhang et al. 2011) supports high heterophil and lymphocyte counts associated with stress, induced by seasonal temperature changes, differences between species, age

and nutrition. Therefore, physiological stress in the form of high heterophil and lymphocytes counts were inferred. Among cohorts, females probably required higher immunity to assist during their reproductive cycles. The high degree of thrombocytes in males was probably elicited by a high requirement for homeostatic regulation. For juveniles perhaps the high degree of lymphocytes was probably related increased growth rates compared to adults.

Despite the fact that haematological studies of reptiles have increased, haematological studies regarding South African tortoises remains a topic of concern. Especially considering habitat destruction, degradation, fragmentation, climate change, and alien invasion can affect the health of tortoises and cause the decline of populations (Paul et al. 2008; Zhang et al. 2011). I recommend further histological research on South African tortoises with particular focus on heterophil to lymphocyte ratios. The latter could be used as a tool to detect physiological stress and disease. Once more comparing the effects of season and cohort with tortoise species, other than *P. geometricus*, was challenging as few chelonian studies specify in which season or age haematological changes occurred.

I have applied histological techniques to describe leukocytes and thrombocytes in the blood profile of *H. areolatus*. I was able to assess the effects of season and cohort on leukocytes and thrombocytes. Furthermore, I have successfully implemented haematology to develop baseline haematological values for *H. areolatus*. In addition to Walton (2012) this is only the second baseline haematological study of tortoises within Southern Africa.

5 GENERAL CONCLUSIONS

Ectothermic reptiles use the ambient environment to regulate their physiological processes (Raske et al. 2012). The physiology of reptiles are therefore affected by environmental fluctuations. These fluctuations are evident in the blood profile of reptiles and can be evaluated by implementation of histological techniques (Paul et al. 2008; Deem et al. 2009). Furthermore intrinsic and extrinsic factors which affect reptilian blood profiles include differences among species, seasonality, age, sex, physiological and nutritional state (Pienaar 1962; Duguy 1970; Strik et al. 2007; Stacy et al. 2011; Zhang et al. 2011; Campbell 2012; Javanbakht et al. 2012; Nardini et al. 2013) A complete histological evaluation includes the assessment of erythrocytes, leukocytes and thrombocytes (platelets in mammals). This study encompassed a histological evaluation with further assessment of the effects of cohort and season on *H. areolatus*.

Upon my evaluation I was able to distinguish and describe immature erythrocytes from mature and senile erythrocytes. I observed evidence to suggest that immature erythrocytes possibly emerged from two distinctive lineages. However, further research is required to discern which lineage gave rise to which immature erythrocyte type. Mature erythrocytes via haemoglobin tetrameters (Strik et al. 2007) play a vital role in gaseous exchange, enabling metabolic functioning for various level of activity (Hartman and Lessler 1964; Dessauer 1970; Walton 2012). Small, mature, ellipsoidal erythrocytes transport oxygen more efficiently than large, spherical erythrocytes.

The morphology of immature erythrocyte types were unaffected by cohort, while cohort effects were evident in mature erythrocytes. Seasonal changes were evident within immature and mature erythrocytes. Erythropoiesis was possibly an indication of nutritional state since it was highest during winter and spring when food and water resources were most abundant. The high incidence of senescence and aberrant features during autumn indicated physiological stress due to limited food and water resources. Mature erythrocytes appeared to be smallest in summer and spring and larger in winter and autumn. Higher temperatures are associated with higher metabolism which allows for more activity, consequently higher energy demands. Therefore, smaller erythrocytes were more prevalent during summer and spring. During the colder months, when temperatures were low, metabolic activity was probably reduced which possibly elicited the prevalence of larger erythrocytes.

Leukocytes in the blood profile of *H. areolatus* included heterophils, eosinophils, lymphocytes, plasma cells, monocytes and azurophils. Additionally thrombocytes were also encountered. The leukocyte profile was affected by cohort and season, such that overall, heterophils appeared to be the most abundant leukocytes in both females and males. The high incidence of heterophils and lymphocytes in females may be linked to a physiological changes elicited by reproduction activities. The fact that eosinophils, basophils and monocytes were present in low frequencies within *H. areolatus* suggests that this tortoise is relatively healthy. However, high heterophil and lymphocyte counts were evident overall within *H. areolatus*. The latter suggests that *H. areolatus* while perhaps proved to be clinically healthy, has endured physiological stress due to limited food availability associated with seasonal temperature changes.

The objectives of this study was met, such that histological techniques enabled me to describe all erythrocyte types and aberrant features, leukocytes as well as thrombocytes . Furthermore, I was able to assess the effects of season and cohort upon blood cell types. Above all, I was able to establish baseline haematological values for *H. areolatus*, which can be used as a reference for future studies to conserve wild and captive ectothermic individuals.

6 REFERENCES

- Adamovicz, L., Bronson, E., Barret, K., Deem, S.L., 2015. Health assessment of free-living Eastern Box turtles (*Terrapene carolina carolina*) in and around the Maryland Zoo in Baltimore 1996-2011. *Journal of Zoo and Wildlife Medicine* 46: 39-51.
- Alexander, G., Marais, J., 2007. *A Guide to the Reptiles of Southern Africa*, First Edition. Struik Publishers, Cape Town, South Africa.
- Aughey, E., Frye, F.L., 2001. *Comparative Veterinary Histology with Clinical Correlates*. Manson Publishing Ltd, London, pp. 51–5870.
- Arikan, H., Çiçek, K., 2010. Morphology of peripheral blood cells from various species of Turkish Herpetofauna. *Acta Herpetologica* 5: 179-198.
- Arikan, H., Çiçek, K., 2014. Haematology of amphibians and reptiles: a review. *North-Western Journal of Zoology* 10: 190-209.
- Arriza, V., Russo, D., Marrone, F., Sacco, F., Arculeo, M., 2014. Morphological characterization of the blood cells in the endangered Sicilian endemic pond turtle, *Emys trinacris* (Testudines: Emydidae). *Italian Journal of Zoology* 81: 344-353.
- Armando, R., Rovira, I., 2010. Hematology of Reptiles, in: Weiss, D.J., Wardrop, K.J., (Eds.), *Schalm's Veterinary Hematology*, sixth edition. Wiley-Blackwell Publishing Ltd, London, pp.1004–1011.
- Atatur, M.K., Arikan, H., Cevik, I.E., Mermer, A., 2001. Erythrocyte measurements of some Scincids from Turkey. *Turkish Journal of Zoology* 25: 149-152.
- Barrows, C.W. 2011. Sensitivity to climate change for two reptiles at the Mojave - Sonoran Desert interface. *Journal of Arid Environments* 75: 629-635.
- Bell, K.A., Gregory, P.T., 2014. White blood cells in Northwestern Gartersnakes (*Thamnophis ordinoides*). *Herpetology Notes* 7: 535-541.
- Bennett, A.F., 1982. The energetics of reptilian activity, in: Gans, C., Pough, F.H. (Eds), *Biology of Reptilia*, Vol B. Academic Press, New York, pp 155-199.
- Bennett A.F., John-Alder, H.B., 1984. The effect of body temperature on the locomotory energetics of lizards. *Journal of Comparative Physiology B* 155: 21-27.
- Bernstein, R.E., 1938. Blood cytology of the tortoise, *Testudo geometrica*. *South African Journal of Science* 35: 327-331.

- Bertolero, A., Nougharede, J-P., Cheylon, M., 2007. Female reproductive phenology in a population of Hermann's tortoise *Testudo hermanni hermanni* in Corsica. *Herpetological Journal* 17: 92-96.
- Boycott, R.C., Bourquin, O., 2000. *The Southern African Tortoise Book*. Hilton, South Africa.
- Branch, W., 1989. *Homopus areolatus*, in: Swingland I.R., Klemens M.W. (Eds.), *The Conservation Biology of Tortoises*. Occasional Papers of the IUCN Species Survival Commission 5: 72-74.
- Brody, T., 2012. Hematopoietic stem cells give rise to the lymphoid lineage and myeloid lineage, in: *Clinical Trials: Study Design, Endpoints and Biomarkers, drug safety and FDA and ICH Guidelines*. Elsevier, San Diego, USA pp. 281-282.
- Brown, T.K., Nagy, K.A., Morafka, D.J., 2005. Costs of growth in tortoises. *Journal of Herpetology* 39: 19-23.
- Bryant, G.L., Flemming, P.A., Twomey, L., Warren, K.A., 2012. Factors affecting hematology and plasma biochemistry in the South Western Carpet Python (*Morelia spilota imbricata*). *Journal of Wildlife Diseases* 48: 282-294.
- Campbell, T.W., 1996 *Clinical Pathology*, in: Mader D.R. (Eds.), *Reptile Medicine & Surgery*. W.B. Saunders Company Ltd Philadelphia, Pennsylvania pp. 248-256.
- Campbell, T.W. 2004. Hematology of lower vertebrates. In: 55th Annual meeting of the American College of Veterinary Pathologists (ACVP) & 39th Annual meeting of the American Society of Clinical Pathology (ASCV), (eds) ACVP & ASCVP, pp. 1214 – 1104. Middleton WI, USA. International Veterinary Information Service, Ithaca, New York (www.ivis.org).
- Campbell, T.W., 2012. Hematology of reptiles, in: Thrall, M.A., Weiser, G., Allison, R.W., Campbell, T.W. (Eds.), *Veterinary Haematology and Clinical Chemistry*. Wiley-Blackwell, Iowa, USA pp. 238-277.
- Campbell, T.W., Ellis, C.K., 2007. *Avian & Exotic Animal Hematology & Cytology*, third edition. Blackwell Publishing, Iowa, pp 51-211.
- Cartledge, V.A., Gartell, B., Jones, S.M., 2005. Adrenal and white cell count responses to chronic stress in gestating and postpartum females of viviparous skink *Egernia whittii* (Scincidae). *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* 141: 100-107.

- Chansue, N., Sailasula, A., Tangtranpiros, J., Wangnaithan, S., 2011. Hematology and clinical chemistry of adult yellow-headed temple turtles (*Hieremys annandalii*) in Thailand. *American Society for Veterinary Clinical Pathology* 40: 174-184.
- Chen, X., Niu, C., Pu, L., 2007. Effects of stocking density on growth and non-specific immune responses in juvenile soft-shelled turtle, *Pelodiscus sinensis*. *Aquaculture Research* 38: 1380-1386.
- Chow, A., Frenette, P.S., 2014 Origin and development of blood cells: Hematopoietic Progenitor Cells, in: Greer, J.P., Arber, D.A., Glader, B., List, A.F., Means, R.T., Jr., Paraskevas, F., Rodgers, G.M., (Eds.), *Wintrobe's Clinical Hematology*. Third Edition. Lippincott Williams and Wilkins, Philadelphia, USA pp. 65-73.
- Christopher, M.M., Berry, K.H., Wallis, I.R., Nagy, K.A., Henen, B.T., Peteron, C.C., 1999. Reference intervals and physiologic alteration in hematologic and biochemical values of free-ranging desert tortoise in the Mojave Dessert. *Journal of Wildlife Diseases* 35: 212-238.
- Crucitti, P., 2012. A review of phenological patterns of amphibians and reptiles in central Mediterranean ecoregion. *Phenology and Climate Change*. www.intechopen.com (Accessed date: 19 August 2014).
- Davis, A.K., Maney, D.L., Maerz, J.C., 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Functional Ecology* 22: 760-772.
- Deem, S.L., Norton, T.M., Mitchell, M., Segars, A.I., Allerman, A.R., Cray, C., Poppenga, R.H., Dodd, M., Karesh, W.B., 2009. Comparison of blood values in foraging, nesting and stranded Loggerhead Turtles (*Caretta caretta*) along the coast of Georgia, USA. *Journal of Wildlife Diseases* 45: 41-56.
- Dessauer, H.C., 1970. Blood chemistry of reptiles: Physiological and evolutionary aspects, in: Gans, C., Parsons, T.S. (Eds.), *Biology of the Reptilia*. Volume 3. Academic Press, London pp. 1-72.
- Dickenson, V.M., Jarchow, J.L., Trueblood, M.H., 2002. Hematology and plasma biochemistry reference range values for free-ranging desert tortoises in Arizona. *Journal of Wildlife Diseases* 35: 143-153.
- Duguy, R., 1970. Number of blood cells and their variations, in: Gans, C., Parsons T.S. (eds), *Biology of the Reptilia*. Volume 3. Morphology C. Academic Press, New York, pp. 93-94.

- Frair, W., 1977. Turtle red blood cell packed volumes, sizes, and number. *Herpetologica* 33: 167-190.
- Fraser, S.T., 2013. The modern primitives: applying new technological approaches to explore the biology of the earliest red blood cells. *ISRN Hematology* 2013: 1-21.
- Frische, S., Bruno, S., Fago, A., Weber, R.E., Mozzarelli, A., 2001. Oxygen binding by single red blood cells from the red-eared turtle *Trachemys scripta*. *Journal of Applied Physiology* 90: 1679-1684.
- Frye, F.L. 1991. Hematology as applied to clinical reptile medicine, in: *Biomedical and Surgical Aspects of Captive Reptile Husbandry*. Second Edition. Volume 1. Krieger Publishing Co., Florida, pp. 209-277.
- Gillooly, J.F., Zenil-Ferguson, R., 2014. Vertebrate blood cell volume increases with temperature: implications for aerobic activity. *PeerJ* 2: e346. doi:10.7717/peerj.346.
- Glomski, C.A., Tamburlin, J., Hard, R., Chainani, M., 1997. The phylogenetic odyssey of the erythrocyte. IV. The amphibians. *Histology and Histopathology* 12: 147-170.
- Gregory, T.R., Andrews, C.B., McGuire, J.A., Witt, C.C., 2009. The smallest avian genomes are found in hummingbirds. *Proceedings of the Royal Society*. www. rspb.royalsocietypublishing.org (Accessed 8 September 2014) DOI: 10.1098/rspb.2009.1004.
- Grenat, P.R., Bionda, C., Salas, N.E., Martino, A.L., 2009. Variation in erythrocyte size between juveniles and adults of *Odontophrynus americanus*. *Amphibia – Reptilia* 30: 141-145.
- Harding, J.M., Torrez-Velez, F., Latimer, K.S., Tarpley, H.L., LeRoy, B.E., 2005. Sea turtle venipuncture and leukocyte morphology. *Veterinary Clinical Pathology Clerkship Program*. (Accessed date: 30 May 2014) <http://www.vet.uqu.edu/vpp/clerk/harding/index.php>.
- Hartman, F.A., Lessler, M.A., 1964. Erythrocyte measurement in fishes, amphibian and reptiles. *Biological Bulletin* 126: 83-88.
- Harvey, J.W., 2012. *Veterinary Hematology: A Diagnostic Guide and Color Atlas* W.B Saunders Elsevier, Missouri, USA pp. 122-193.
- Hatami, K., Sayyadi, F., Parto, P., Yousefan, N., Rastegar-Pouyani, N., 2014. Evaluating the size of erythrocytes in the blood of *Acanthodactylus nilsoni* (Sauria: Lacertidae) from Iran. *World Journal of Zoology* 9: 80-85.

- Hawkey, C.M., Dennet, T.B., 1989. A Colour Atlas of Comparative Veterinary Hematology. Wolfe Medical Publishers Limited, London, pp. 9-15
- Henen, B.T., 1997. Seasonal and annual energy budgets of female desert tortoise (*Gopherus Agassizii*). *Ecology* 78: 283-296.
- Hofmeyr, M.D., 2004. Egg production in *Chersina angulata*: An unusual pattern in a Mediterranean climate. *Journal of Herpetology* 38: 172-179.
- Hofmeyr, M.D., Boycott, R.C., Baard, E.H.W., 2014. Family Testudinidae, in: Bates, M.F., Branch, W.R., Bauer, A.M., Burger, M., Marais, J., Alexander, G.J., de Villiers, M.S., (Eds.), Atlas and Red List of the Reptiles of South Africa, Lesotho and Swaziland. South African Biodiversity Institute, Pretoria pp. 70-85.
- Homyack, J.A., Haas, C.A., Hopkins, W.A., 2010. Influence of temperature and body mass on standard metabolic rate of eastern red-backed salamanders (*Plethodon cinereus*). *Journal of Thermal Biology* 35: 143-146.
- Honegger, R.E., 1979. Marking amphibians and reptiles for future identification. *International Zoo Yearbook* 19: 14-22.
- Houwen, B., 2000. Blood film preparation and staining procedures. *Laboratory Haematology* 6: 1-7.
- Irwin, M.T., Junge, R.E., Raharison, J-L., Samonds, K.E., 2010. Variation in physiological health of Diademed Sifakas across intact and fragmented forest at Tsinjoarivo, Eastern Madagascar. *American Journal of Primatology* 72: 1013-1025.
- Jacobson, E.R., 1994. Causes of mortality and diseases in tortoises: A review. *Journal of Zoo and Wildlife Medicine* 25: 2-17.
- Jacobson, E.R., 2007. Overview of reptile biology, anatomy, and histology, in: *Infectious Diseases and Pathology of Reptiles*. CRC Press, Taylor & Francis Group, Boca Raton, Florida pp. 19-20.
- Jackson, D.C., 2007. Temperature and hypoxia in ectothermic tetrapods. *Journal of Thermal Biology* 32: 125-133.
- Javanbakht, H., Vaissi, S., Parto, P., 2013. The morphological characterization of the blood cells in three species of turtle and tortoises in Iran. *Research in Zoology* 3: 38-44.
- Jensen, F.B., 2009. The dual roles of red blood cells in tissue oxygen delivery: oxygen carriers and regulators of local blood flow. *The Journal of Experimental Biology* 212: 3387-3393.

- Johnstone, C.P., Lill, A., Reina, R.D., 2012. Does habitat fragmentation cause stress in the agile antechinus? A haematological approach. *Journal of Comparative Physiology B* 182: 139-155.
- Jordaan, H.E., 1938. Comparative hematology (Reptilia), in: Downey, H. (Eds), *Handbook of hematology*. P.B. Hoeber Inc., New York, Vol II, 776-788.
- Joshua, Q.I., Hofmeyr, M.D., Henen, B. T., 2010. Seasonal and site variation in angulate tortoise diet and activity. *Journal of Herpetology* 44: 124-134.
- Kassab, A., Shousha, S., Fargani, A., 2009. Morphology of blood cells, liver and spleen of the desert tortoise (*Testudo graeca*). *The Open Anatomy Journal* 1: 1-10.
- Keswick T., 2012. Ecology and morphology of the Kalahari tent tortoise, *Psammobates oculifer*, in a semi-arid environment. Master Thesis. Univeristy of the Western Cape, South Africa.
- Keswick, T., Henen, B.T., Hofmeyr, M.D., 2006. Sexual disparity in activity patterns and time budgets of angulate tortoises (*Chersina angulata*) on Dassen Island, South Africa. *African Zoology* 41: 224-233.
- Khanna, D.R., Yadav, P.R., 2005. *Biology of Mammals*. Discovery Publishing House, New Delhi, India pp. 210-262.
- Kingsley, P.D., Malik, J., Fantauzzo, K.A., Palis, J., 2004. Yolk Sac-derived primitive erythroblasts enucleate during mammalian embryogenesis. *Blood* 140: 19-25.
- Knotková, Z., Doubek, J., Knotek, Z., Hajkova, P., 2002. Blood cell morphology and plasma biochemistry in Russian tortoises. *Acta Vet. BRNO* 71: 191-198.
- Knotek, Z., Knotková, Z., Trnková, S., 2006. Advances in reptilian haematology and blood chemistry. *Proceedings of the 31st World Small Animal Association Congress, 12th European Congress FECAVA, & 14th Czech Small Animal Veterinary Association Congress, Prague, Czech Republic*, pp. 334-336.
- Lagarde, F., Bonnet, X., Nagy, K., Henen, B., Legrand, A., Corbin, J., Naulleau, G., 2002. A short spring before a long jump: the ecological challenge to the steppe tortoise (*Testudo horsfieldi*). *Canadian Journal of Zoology* 81: 380-387.
- Lisičić, D., Đikić, D., Benković, V., Knežević, A.H., Oršolić, N., Tadić, Z., 2013. Biochemical and hematological profiles of a wild population of the nose-horned viper *Vipera ammodytes* (Serpentes: Viperidae) during

- autumn, with a morphological assessment of blood cells. *Zoological Studies* 52: 2-9.
- Litzgus, J.D., Hopkins, W.A., 2003. Effect of temperature on metabolic rate of the mud turtle (*Kinosternon subrubrum*). *Journal of Thermal Biology* 28: 595-600.
- Loehr, V.J.T., 2002. Population characteristics and activity patterns of the Namaqualand Speckled Padloper (*Homopus signatus signatus*) in the early spring. *Journal of Herpetology* 36: 378-389.
- Loehr, V.J.T., 2012. High body temperature in an arid, winter-rainfall environment: Thermal biology of the smallest tortoise. *Journal of Arid Environments* 82: 123-129.
- Loehr, V.J.T., Hofmeyr, M.D., Henen, B. T., 2009. Small and sensitive to drought: consequences of aridification to the conservation of *Homopus signatus signatus*. *African Journal of Herpetology* 58: 116-125.
- Longshore, K.M., Jaeger, J.R., Sappington, M.J., 2003. Desert tortoise (*Gopherus agassizii*). Survival at two Eastern Mojave Desert sites: Death by short-term drought? *Journal of Herpetology*, 37: 169-177.
- López-Ortiz, R., Lewis, A.R., 2004. Habitat selection by *Sphaerodactylus nicholsi* (Squamata gekkonidae) in Cabo Rojo, Puerto Rico. *Herpetologica* 60: 438-444.
- Maciak, S., Kostelecka-Myrcha, A., 2011. Regularities of variation of the red blood indices characterizing the respiratory function of blood in selected fish. *Zoologica Poloniae* 56: 35-48.
- Mader, S., 1997. Circulatory system: Blood, in: Mills, C.J., Balian-Haakinson, C., Horn, M.B., Banowitz, J.K., Hancock, L. (Eds.), *Inquiry into Life*. McGraw-Hill Companies, Missouri, USA pp190-240.
- Mader, D.R., 2000. Normal hematology of reptiles, in: Feldman, B.F., Zinkl, J.G., Jain, N.C. (Eds.), *Schalm's Veterinary Hematology*. Lippincott Williams and Wilkins, Philadelphia, USA pp. 1126-1132.
- Maekawa, S., Lemura, H., Kuramochi, Y., Nogawa-Kosaka, N., Nishikawa, H., Okui, T., Aizawa, Y., Kato, T., 2012. Hepatic confinement of newly produced erythrocytes caused by low-temperature exposure in *Xenopus laevis*. *The Journal of Experimental Biology* 215: 3087-3095.
- Mayer, J., Donnelly, T.M., 2013. Laboratory test: basophil count, in: *Clinical Veterinary Advisor: Birds and Exotic pets*. W.B. Saunders Elsevier, Missouri, USA pp. 604.

- Martinho, F. 2012. Blood Transfusion in Birds. *Revista Lusófona de Ciência e Medicina Veterinária* 5: 1-30.
- Martinez-Agosto, J.A., Mikkola, H.K.A., Hartenstein, V., Banerjee, U., 2007. The hematopoietic stem cell and its niche: a comparative view. *Genes and Development* 21: 3044-3060.
- McGeady T.A., Quinn, P.J., FitzPatrick, E.S., Ryan, M.T., 2006. *Veterinary Embryology*. Blackwell Publishing Ltd, Oxford, UK.
- Metin, K., Koca, Y.B., Kiral, F.K., Koca, S., Türkozan, O., 2008. Blood cell morphology and plasma biochemistry of captive *Mauremys caspica* (Gmelin 1774) and *Mauremys rivulata* (Valenciennes 1833). *Acta Vet. BRNO* 77: 163-174.
- Mendoza-Rangel, J., Weber, M., Zenteno-Ruiz, C.E., Lopez-Luna, M.A., Barba-Macias, E., 2009. Hematology and serum biochemistry comparison in wild and captive Central American river turtles (*Dematemys mawaii*) in Tabasco, Mexico. *Research in Veterinary Science* 87: 313-318.
- Mitchell, N.J., Jones, T.V., Kuchling, G., 2012. Simulated climate change increases juvenile growth in a critically endangered tortoise. *Endangered Species Research* 17: 73-82.
- Moreno-Rueda, G., Pleguezuelos, J.M., Alaminos, E., 2009. Climate warming and activity period extension in the Mediterranean snake *Malpolon monspessulanus*. *Climatic Change* 92: 235-242.
- Motlagh, S.P., Zarejabad, A.M., Nasrabadi, R.G., Ahmadifar, E., Molaee, M., 2010. Haematology, morphology and blood cells characteristics of male and female Siamese fighting fish (*Betta splendens*). *Comparative Clinical Pathology* Online publication: http://scientificfinding.gau.ac.ir/uploading/scientificfinding.gau.ac.ir/images/ghorbani_rasool/poorali.pdf. DOI10.1007/s00580-010-1058-6. Published: 13 July 2010. (Accessed 8 September 2014).
- Moulherat, S., Delmas, V., Slimani, T., E.L., Hassen, Mouden, E.L., Louzizi, T., Lagarde, F., Bonnet, X., 2014. How far can a tortoise walk in open habitat before overheating? Implications for conservation. *Journal for Nature Conservation* 22: 186-192.
- Muñoz, F.J., De la Fuente, M., 2004. Seasonal changes in lymphoid distribution of the turtle *Mauremys caspica*. *Copeia* 1: 178-183.
- Nabity, M.B., Ramaiah, S.K., 2012. Blood and bone marrow toxicology: hematopoietic stem cells, in: Gupta, R.C. (Eds.), *Veterinary Toxicology: Basic and*

- Clinical Principles. Academic Press, Elsevier, Waltham, Massachusetts pp. 352-354.
- Nardini, G., Leopardi, S., Bielli, M., 2013. Clinical hematology in reptilian species. *Veterinary Clinics of North America: Exotic Animal Practice* 16: 1-30.
- Nussey, D.H., Froy, H., Lemaitre, J-F., Gaillard J-M., Austad, S.N., 2013. Senescence in natural populations of animals: Widespread evidence and its implications for bio-gerontology. *Ageing Research Reviews* 12: 214-225.
- Oliveira-Júnior, A.A, Tavares-Dias, M., Marcon, J.L., 2009. Biochemical and hematological reference ranges for Amazon freshwater turtle, *Podocnemis expansa* (Reptilia: *Pelomedusidae*), with morphologic assessment of blood cells. *Research in Veterinary Science* 86: 146-151.
- Otis, V.S., 1973. Hemocytological and serum chemistry parameters of the African puff adder, *Bitis arietans*. *Herpetologica* 29: 110-116.
- Overgaard, J., Wang, T., 2002. Increased blood oxygen affinity during digestion in the snake *Python molurus*. *The Journal of Experimental Biology* 205: 3327-3334.
- Parida, S.P., Dutta, S.K., Pal, A., 2014. Hematology and plasma biochemistry of wild-caught Indian cobra *Naja naja* (Linnaeus 1758). *Journal of Venomous Animals and Toxins including Tropical Diseases* 20: 1-7.
- Parto, P., Rastegar-Pouyani, N., Vaissi S., Zarei, F., Karamiani, R., 2013. Erythrocyte size of some snake species from West of Iran (*Platycephalus najadum najadum*, *Malpolon insignitus insignitus* and *Eirenis collaris*) after hibernation. *World Journal of Zoology* 8: 324-327.
- Paul, M.J., Zucker, I., Schwartz, W.J., 2008. Tracking the seasons: the internal calendars of vertebrates. *Philosophical Transactions of the Royal Society B* 363: 341- 361.
- Pendl, H., 2006. Morphological changes in red blood cells of birds and reptiles and their interpretation. *Israel Journal of Veterinary Medicine* 61: 1-12.
- Perpiñán, D., Hernandez-Divers, S.M., Latimer, K.S., Akre, T., Hagen, C., Buhlmann K.A., Hernandez-Divers, S.J., 2008. Hematology of the Pascagoula map turtle (*Graptemys gibbonsi*) and the Southeast Asian box turtle (*Cuora amboinensis*). *Journal of Zoo and Wildlife Medicine* 39: 460-463.

- Peterson, C.C., 2002. Temporal, population, and sexual variation in haematocrit of free-living desert tortoises: correlational tests of causal hypotheses. *Canadian Journal of Zoology* 80: 461-470.
- Pienaar, U de V., 1962. Haematology of some South African reptiles. Witwatersrand University Press, Johannesburg, South Africa pp. 35-60.
- Pough, H., 1980. Blood oxygen transport and delivery in reptiles. *American Zoologist* 20: 173-185.
- Quigley, J.G., Means, R.T., Glader, Jnr. B., 2014. The erythrocyte, in: Greer, J.P., Arber, D.A., Glader, B., List, A.F., Means Jnr. R.T., Paraskevas, F., Rodgers, G.M. (Eds.), *Wintrobe's Clinical Hematology*. Thirteenth Edition. Lippincott Williams and Wilkins, Philadelphia, USA pp. 83-85.
- Quinn, G.P., Keough, M.J., 2002. *Experimental Design and Data Analysis for Biologists*. Cambridge University Press, UK pp. 339-441.
- Ramsay, S.L., Hofmeyr, M.D., Joshua, Q.I., 2002. Activity patterns of the angulate tortoise (*Chersina angulata*) on Dassen Island, South Africa. *Journal of Herpetology* 36: 161-169.
- Raske, M., Lewbart, G.A., Dombrowski, D.S., Hale, P., Correa, M., Christian, L.S., 2012. Body temperature of selected amphibian and reptile species. *Journal of Zoo and Wildlife Medicine* 43: 517-521.
- Reavill, D. 1994. Selected Topics in Reptile Clinical Pathology. Lecture given at the U.C.Davis Avian/Exotic Animal Symposium 12 pp, <http://www.zooexotic.com/Reptileclinpath1994.pdf> (Accessed 24 July 2014).
- Rios, F.S., Oba, E.T., Fernandes, M.N., Kalinin, A.L., Rantin, F.T., 2005. Erythrocyte senescence and haematological changes induced by starvation in neotropical fish traíra, *Hoplias malabaricus* (Characiformes, Erythrinidae). *Comparative Biochemistry and Physiology, Part A* 140: 281-287.
- Rombough, P., Drader, H., 2009. Hemoglobin enhances oxygen uptake in larval zebrafish (*Danio rerio*) but only under conditions of extreme hypoxia. *The Journal of Experimental Biology* 212: 778-784.
- Rossini, M., Garcia, G., Rojas, J., Zerpa, H., 2011. Hematologic and serum biochemical reference values for the wild spectacled caiman, *Caiman crocodilus, crocodilus*, from the Venezuelan plains. *Veterinary Clinical Pathology* 40: 374-379.
- Rouget, M., Reyers, B., Jones, Z., Desmet, P., Driver, A., Maze, K., Egoh, B., Cowling, R.M., 2004. *South African National Spatial Biodiversity*

- assessment 2004, technical report, Volume 1 terrestrial component. South African National Biodiversity Institute, Pretoria, pp. 1-78.
- Russel, K.E., 2010. Platelet kinetics and laboratory evaluation of thrombocytopenia, in: Weiss, D.J., Wardrop, K.J. (Eds.), *Schalm's Veterinary Hematology*, sixth edition. Wiley-Blackwell, Iowa, USA pp. 576-577.
- Sano-Martins, I.S., Dabrowski, Z., Tabarowski, Z., Witkowaska-Pelc, E., Morena, D.D.S., Spodaryk, K., 2002. Haematopoiesis and a new mechanism for the release of mature blood cells from the bone marrow into the circulation in snakes (Ophidia). *Cell Tissue Research* 310: 67-75.
- Sacchi, R., Pupin, F., Zuffi, M.A.L., Scali, S., Boncompagni, E., Binda, A., Galeotti, P., Fasola, M., 2007. Blood cell morphology of Moorish gecko, *Tarentola mauritanica*. *Amphibia-Reptilia* 28: 503-508.
- Setlalekgomo, M.R., Winter, P.E.D., Els, S.F., 2012. The metabolic adjustments of the angulate tortoise (*Chersina angulata*) to seasonal changes in temperature and photoperiod. *Journal of Applied Sciences Research* 8: 1211-1218.
- Scantlebury, M., Minting, P., 2006. Differences in resting metabolic rates of two Southern African tortoises: *Psammobates oculiferus* and *Geochelone pardalis*. *African Journal of Herpetology* 55: 161-165.
- Schneck, D.J., 2003. An outline of cardiovascular structure and function: the working fluid: blood, in: Mudry, K.M., Plonsey, R., Bronzino, J.D. (Eds.), *Principles and Applications in Engineering Series*. Volume 1. CRC Press LLC, Florida, USA pp. 1-4.
- Schwanz, L., Warner, D.A., McGaugh, S., Terlizzi, R.D., Bronikowski, A., 2011. State-dependent physiological maintenance in a long-lived ectotherm, the painted turtle (*Chrysemys picta*). *The Journal of Experimental Biology* 214: 88-97.
- Shadkhast, M., Shabazkia, H-R., Sadegh, A.B., Shariati, S.E., Mahmoudi, T., 2010. The morphological characterization of the blood cells in the Central Asian tortoise (*Testudo horsfieldii*). *Veterinary Research Forum* 1: 134-141.
- Sheridan, J.A., Bickford, D., 2011. Shrinking body size as an ecological response to climate change. *Nature Climate Change* 1: 401-405.
- Snyder, G.K., Sheafor, B.A., 1999. Red blood cells: Centerpiece in the evolution of the vertebrate circulatory system. *American Zoologists* 39: 189-198.

- Stacy, B.A., Whitaker, N., 2000. Hematology and blood biochemistry of captive mugger crocodiles (*Crocodylus palustris*). *Journal of Zoo and Wildlife Medicine* 31: 339-347.
- Stacy, N.I., Alleman, A.R., Saylor, K.A., 2011. Diagnostic haematology of reptiles. *Clinics in Laboratory Medicine* 31: 87-108.
- Stawski, C.Y., Grigg, G.C., Booth D.T., Beard, L.A., 2006. Temperature and respiration properties of whole blood in two reptiles, *Pogona barbata* and *Emydura signata*. *Comparative Biochemistry and Physiology, Part A* 143: 173-183.
- Stevenson, R.D., 1985. Body size and limits to the daily range of body temperature in terrestrial ectotherms. *The American Naturalist* 125: 102-117.
- Steward, J.R., Florian, J.D., Jr., 2000. Ontogeny of the extraembryonic membranes of the oviparous lizard, *Eumeces fasciatus* (Squamata Scincidae). *Journal of Morphology* 244: 81-107.
- Strik, N.I., Alleman, A.R., Harr, K.E., 2007. Circulating inflammatory cells, in: Jacobson, E.R. (Ed.), *Infectious Diseases and Pathology of Reptiles*. CRC Press, Taylor & Francis Group, Boca Raton, Florida pp. 167-218
- Starostova, Z., Konarzewski, M., Koziowski, Kozłowski, J., Kratochvil, L., 2013. Ontogeny of metabolic rate and red blood cells size in eyelid geckos: species follow different paths. *the PLoS One* 8(5): e64715. doi:10.1371/journal.pone.0064715.
- Sypek, J., Borysenko, M., 1988. Hematopoiesis, in: Rowley, A.F., Ratcliffe, N.A. (Eds.), *Vertebrate Blood Cells*. Cambridge University Press, UK pp. 214-216.
- Theml, H., Diem, H., Haferlach, T., 2004. Normal cell of blood and hematopoietic organs: lymphocytes (and plasma cells), in: *Colour Atlas of Hematology: Practical Microscopic and Clinic Diagnosis*. Clinical Science. Georg Thieme Verlag, Stuttgart, Germany pp. 45-48.
- Tkachuk, D.C., Hirschmann, J.V., McArthur, J.R., 2002. *Atlas of Clinical Hematology*. W.B. Saunders publishers, Philadelphia, pp. 4–11.
- Torres, J.J., Grigsby, M.D., Clarke, M.E., 2012. Aerobic and anaerobic metabolism in oxygen minimum layer fishes: the role of alcohol dehydrogenase. *The Journal of Experimental Biology* 215: 1905-1914.
- Torsoni, M.A., Stoppa, G.R., Turra, A., Ogo, S.H., 2002. Functional behaviour of tortoise hemoglobin *Geochelone denticulata*. *Brazilian Journal of Biology* 62: 725-733.

- Tosunoğlu, M., Tok, C.V., Gul, C., 2005. Hematological values in Hermann's tortoise (*Testudo hermanni*) and spur-thighed tortoise (*Testudo graeca*) from Thrace Region (Turkey). *International Journal of Zoological Research* 1: 11-14.
- Tripathi, M.K., Singh, R., 2014. Melatonin modulates splenocyte immune response in the freshwater snake, *Natrix piscator*. *Herpetological Conservation and Biology* 9: 257-266.
- Uğurtaş, I.H., Sevinc, M., Yildirimhan, H.S., 2003. Erythrocyte size and morphology of some tortoises and turtles from Turkey. *Zoological Studies* 42: 173-178.
- Vandegriff, K.D., Olson, J.S., 1984. Morphological and physiological factors affecting oxygen uptake and release by red blood cells. *The Journal of Biological Chemistry* 259: 12619-12627.
- van Bloemestein, U.P., 2005. Seasonal movement and activity patterns of endangered geometric tortoise, *Psammobates geometricus*. Masters Thesis. University of the Western Cape, South Africa. (Unpublished).
- Vasse, B.J., Beaupain, D., 1981. Erythropoiesis and haemoglobin ontogeny in the turtle *Emys orbicularis* L. *Journal of Embryological & Experimental Morphology* 62: 129-138.
- Walton, S., 2012. Effect of season and cohort on the haematology of the geometric tortoise *Psammobates geometricus*. Masters Thesis. University of the Western Cape, South Africa (Unpublished).
- Walton, S., Hofmeyr, MD., van der Horst, G., 2012. Accurate automated quantitative imaging of tortoise erythrocytes using the NIS image analysis system. *Biotechnic & Histochemistry* 88: 242-249.
- Weber, R.E., 2007. High-altitude adaptations in vertebrate hemoglobins. *Respiratory Physiology & Neurobiology* 158: 132-142.
- Weiser, G., 2012. Laboratory techniques for veterinary medicine: hematologic techniques, in: Thrall, MA., Weiser, G., Allison, R., Campbell, T. (Eds.), *Veterinary Haematology and Clinical Chemistry*. Blackwell Publishing, London, pp. 3-8.
- Wilson, D.S., Morafka, D.J., Tracy, C.R., Nagy, K.A., 1999. Winter activity of juvenile desert tortoises (*Gopherus agassizii*) in the Mojave Desert. *Journal of Herpetology* 33: 496-501.
- Windberger, U., Baskurt, O.K., 2007. Comparative hemorheology. *Handbook of Hemorheology and Hemodynamics*. IOS Press, Amsterdam, Netherlands pp 267-285.

- Wood, S.C., 1980. Adaptation of red blood cell function to hypoxia and temperature in ectothermic vertebrates. *American Zoologist* 20: 163-172.
- Wojtaszek, J., Adamowicz, A., 2003. Haematology of the fire-bellied toad, *Bombina bombina* L. *Comparative Clinical Pathology* 12: 129-134.
- Work, T.M., Raskin, R.E., Balazs, G.H., Whittaker, S.D., 1998. Morphological and cytochemical characteristics of blood cells from Hawaiian green turtles. *American Journal of Veterinary Research* 59: 1252-1257
- Yu, P-H., Yang, P-Y., Chiu, Y-S., Chi, C-H., 2013. Hematologic and plasma biochemical reference values of the yellow pond turtle *Mauremys mutica* and effects of sex and season. *Zoological Studies* 52: 1-6.
- Zhang, F., Hexiang, G.U., Pipeng, L.I., 2011. A review of chelonian haematology. *Asian Herpetological Research* 2: 12-20.
- Zimmernan, L.C., O'Connor, M.P., Bulova, S.J., Spotila, J.R., Kemp S.J., Salice, C.J., 1994. Thermal ecology of desert tortoises in the Eastern Mojave Desert: Seasonal patterns of operative and body temperatures, and microhabitat utilization. *Herpetological Monographs* 8: 45-59.

