Effects of season and cohort on the haematology of the geometric tortoise *Psammobates geometricus*

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KEYWORDS

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ABSTRACT

The geometric tortoise is one of the world's rarest terrestrial tortoises and is endemic to the Southwestern Cape, South Africa. There has been cause for conservation concern for *Psammobates geometricus*, yet as is common for many species, quantitative physiological research has been lacking. Considering the important role of red blood cells in oxygen circulation, and the role of white blood cells in immune resistance, blood profiles have been used across taxa as a reliable indicator of health status and physiological processes. Forming part of a larger chelonian conservation programme in South Africa, I studied the haematological changes in *P. geometricus*, to better understand their physiological responses to changes in climatic conditions. I sampled peripheral blood from males, females and juveniles of the largest known wild geometric tortoise population over four seasons (spring, summer, autumn and winter) from August 2000 to June 2001. Blood samples were used to make smears and determine red cell count (RCC), packed cell volume (PCV), haemoglobin concentration (Hb), red cell indices and differential white cell counts. Digital imaging analysis was used for the histological evaluation of stained blood smears, including descriptions of red and white blood cell morphologies, as well as erythrocyte developmental stages. In the cooler periods, geometric tortoises showed low Hb and mean cell haemoglobin concentration values. Erythrocytes were larger and rounder in winter and spring, which were likely due to hydration states. In addition, increased numbers of immature erythrocytes in circulation suggested an erythropoietic response in winter and spring. This regenerative response is common in reptiles emerging from periods of limited activity and is associated with increasing primary production following rainfall events. In the following summer and autumn, increased mean cell haemoglobin concentrations suggested elevated metabolic rates influenced by rising temperatures. This would seem pertinent to meet the extra physical demands associated with foraging effort in the season characterised with limited water and food supply, and mating behaviour, which occurs in the summer. Low body conditions across all cohorts provided evidence for nutrition stress, while erythrocyte size, shape and degenerative responses indicated dehydration stress. Physiological responses to seasonal influences are specific to growth or reproductive demands and differed for each cohort. Males experienced increased Hb, PCV, RCC, and erythrocyte sizes in summer and autumn, which relate to the erythropoietic-stimulating effects of androgens. Female erythropoietic cycles in spring accommodate the increased metabolic demands of increased foraging needed for a

larger body size and egg production, and again in autumn again for vitellogenesis. Juvenile tortoises showed minimal differences, and could indicate species-specific responses to environmental changes. A spring-related erythropoiesis was observed in juveniles while during summer and autumn, juveniles showed less evidence for dehydration stress than in adults. No haemoparasites were observed in peripheral blood. Seven leukocyte types were identified and included heterophils, eosinophils, basophils, lymphocytes, plasma cells, monocytes and azurophils, in addition to thrombocytes. Heterophils were the most abundant leukocyte, followed by lymphocytes and eosinophils while monocytes and basophils were equally low; plasma cells and azurophils were rare. Heterophil counts were higher in spring than in summer and autumn, and in summer, were more abundant in females than in juveniles. Eosinophil counts were low in spring for all cohorts, and additionally, female and juvenile counts were low in summer. Eosinophils in juveniles were significantly lower than in adults in winter and spring. Lymphocyte numbers increased in autumn for all cohorts, while summer counts were higher in juveniles than in adults. Basophils and monocytes showed minimal seasonal changes, although basophil counts in females in winter tended to be high. Thrombocytes were lowest in spring for all cohorts. Understanding the physiological responses associated with seasonal changes and for each cohort is critical for effective chelonian conservation management. Results obtained from this study indicate a clinically healthy population of *Psammobates geometricus* and represented the first of this kind to establish baseline haematological reference data for this Critically Endangered tortoise species.

August 2012

DECLARATION

I declare that Effects of season and cohort on the haematology of the geometric tortoise *Psammobates geometricus* 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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Shasheen Walton August 2012

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1 GENERAL INTRODUCTION

1.1 BLOOD OF REPTILES

Similar to the higher vertebrates, blood cells in the peripheral blood of reptiles consist of erythrocytes (red blood cells, RBC), leukocytes (white blood cells) and thrombocytes. While reptilian erythrocytes exceed avian and mammalian red blood cells in size, their counts are lower than those of birds and mammals (Reavill 1994).

Due to their ectothermic nature, reptilian physiology (and therefore numbers and types of circulating blood cells) is more influenced by external, environmental conditions than those of endothermic vertebrates. Specific to reptile blood, certain cells remain pluripotent in the peripheral bloodstream, often making them difficult to identify. A complete evaluation of the haemogram involves determination of total red cell count (RCC), packed cell volume (PCV), haemoglobin concentration (Hb), white cell counts (total and differential) and the histological evaluation of a stained peripheral blood film, including interpretation of the blood cell morphology.

1.1.1 Erythrocytes

In contrast to mammalian erythrocytes, reptilian erythrocytes are true nucleus-containing cells. Since the primary role of erythrocytes is to transport haemoglobin that carries oxygen to the tissues, the size, shape and volume of erythrocytes serve as an indication of the surface area available for gaseous exchange to meet respiratory demands (Hartman & Lessler 1964). The red blood cell is involved in the determination of PCV (an indication of erythrocyte mass), RCC (number of erythrocytes in circulation) and Hb (oxygen carrying capacity). Typically, reptiles have a lower RCC than that of birds and mammals and there is an inverse relationship between the size and total number of circulating erythrocytes (Saint Girons 1970; Frair 1977). Erythrocyte life cycle compared to that of mammals, the lifespan of the reptilian erythrocyte (600 – 800 days) has a long turnover rate, due to the slower metabolic rate of reptiles (Mader 2000).

Erythrocyte morphology in blood smears is central to the haematological investigation. In light of the clear functional role of the red blood cells in oxygen transport and delivery, in particular erythrocyte size, shape, and colour become informative. Morphological variation is especially indicative of anaemic conditions; variation in red blood cell size is anisocytosis, variation in red cell shape includes

poikilocytosis, variation in red cell colour includes polychromasia (increased numbers of immature red cells) and hypochromasia (reduced haemoglobin in red cells; Pendl 2006).

1.1.2 Leukocytes

Typically observed in the blood smear are the granulocytes and agranulocytes, characterised by the presence (or absence) of specific granules that identifies the cell lineage (Harding *et al*. 2005). The granulocytes include heterophils, eosinophils and basophils; the agranulocytes include lymphocytes, plasma cells and monocytes, while azurophils are considered monocytic leukocytes by most authors. Leukocytes perform the same roles in reptiles as in mammals, with some minor differences. The leukogram includes calculation of the total leukocyte count, determination of the differential leukocyte count, and evaluation of the overall cellular morphology. As with erythrocytes, manual methods for determination of total leukocyte counts are performed. The most common manual methods include the direct method (using Unopette system or Natt and Herrick solution) or the estimate method in which total number of leukocytes are counted in each field for ten fields on a blood smear and an average taken. To determine the differential leukocyte count, 100 leukocytes are counted and the cell type recorded to determine the relative percentage of leukocyte (Harding *et al.* 2005). The classification criteria of chelonian leukocytes vary among studies as some cells are not easily identified on the basis of their morphological differences alone.

The heterophil is commonly referred to the mammalian neutrophil equivalent and is the most numerous leukocyte (Harding *et al*. 2005). While their primary role is phagocytosis, heterophils respond to tissue inflammation and infection (Mader 2000). It is common to observe all three forms (mature, immature, toxic) in the blood smear.

The reptilian eosinophil behaves like that of avians (Reavill 1994) and is as distinct as in mammals (Mader 2000). In reptiles, they react to parasitic stimuli (external and blood parasites) and although a true eosinophlilic response is not observed (Reavill 1994), it is reported that in chelonians, they act in immune response and phagocytose antigens (Mader 2000).

The basophil is a readily identifiable cell in the reptilian blood smear and similar to mammals, basophils are believed to be involved in the processing of surface immunoglobulins and histamine release. The leukocyte showing the least seasonal

differences, basophils may be affected by the presence of blood parasites (Mader 2000).

The lymphocyte is often the second most abundant leukocyte in the blood smear (Mader 2000; Harding *et al.* 2005) and originates from the thymus, bone marrow, spleen and other lymphopoietic tissues. These cells are believed to retain their pluripotency while they are circulating in the blood (Saint Girons 1970). In addition, they produce certain immunoglobulins as well as moderate immune response. Lymphocyte counts are believed to be lower in males and under starving conditions and lymphocytosis can occur in cases of inflammation, wound healing and certain parasitic infections (Mader 2000). Plasma cells or plasmacytes, as in mammals, represent an antibody-producing B cell lymphocyte in response to a specific antigen, the effects of which are short-lived (microbiology glossary; www.library.thinkquest.org).

The monocyte is typically the largest and least abundant leukocyte (Reavill 1994), the reptilian monocyte is similar to its mammalian counterpart, and shows little seasonal variation. Numbers are affected by antigenic stimulation; monocytes are involved in bacterial infection (Reavill 1994) and also play an active role in granuloma formation (Mader 2000). Azurophils have been reported in chelonian circulating blood, and are considered by many haematologists to represent a type of monocyte (Christopher *et al.* 1999; Knotkova *et al*. 2002; Dickinson *et al.* 2002).

1.1.3 Thrombocytes

Similar to the mammalian platelet, these cells are involved with blood clot formation, wound healing and thrombosis (Mader 2000). Reptilian thrombocytes play a role in haemostasis (Campbell 2004). As in avian blood, reptilian thrombocytes are nucleated cells.

While it is widely accepted that all peripheral blood cells arise from one multipotent haemoblast capable of differentiating in the bone marrow or spleen into the various blood cells, Saint Girons (1970) identifies two possibilities that postulate different origins. One begins with the haemoblasts for the leukocytic series on one lineage and for the erythrocytes on the other. Another possibility is that blood cells have three distinct origins: monocytes derive from a stem cell of the reticulo-endothelial system;

granulocytes, and thrombocytes arise from a second type of stem cell, the myeoblast; and finally the erythrocytes arise from the erythroblasts.

1.2 FACTORS INFLUENCING HAEMATOLOGICAL PARAMETERS

Many factors influence the haematological parameters of reptiles, including age, sex, season, environmental conditions, health, level of exercise and even circulating hormones of individuals (Frair 1977; Reavill 1994; Christopher *et al.* 1999; Mader 2000; Dickinson *et al.* 2002).

Within species, haematological differences have been reported between juveniles and adults (Mader 2000; Knotek *et al*. 2006; Casal & Oros 2007), and are related to increased growth before the onset of sexual maturity (Kuchling 1999). Differences also arise between males and females (Gardner & Gorshein 1973; Frair 1977) and are related to differing reproductive hormones, strategies and timing (Anderson *et al*. 1997; Henen *et al.* 1998; Peterson 2002). Owing to their ectothermic nature, environmental and seasonal differences in blood values have been widely recorded for chelonian species (Anderson *et al*. 1997; Christopher *et al*. 1999; Knotek *et a*l. 2006). Dickinson (2002) attributed seasonal haematology differences in the Desert tortoise to rainfall, forage availability as well as physiological condition. The effects of environmental conditions including polluted habitats on haematology have been studied in freshwater turtles (Ferronato *et al*. 2009; Tosunoglu *et al*. 2011). Oyewale *et al*. (1998) compared haematological differences between two species in identical environmental conditions (*Kinixys erosa* & *Gopherus agassizii*). Haematological differences in captive chelonians have also been studied in several species (Martinez-Silvestre *et al.* 2001; Brenner *et al.* 2002; Metin *et al.* 2008).

Compared to mammals, the concentration of reptile blood constituents and plasma fluctuate more due to feeding, temperature changes and water availability (Dessauer 1970). Tortoises are opportunistic osmoregulators, and under drought conditions, body mass and total body water volume declines. Consequently, blood plasma osmolality increases (a condition called haemoconcentration), and in *G. agassizii* (Peterson 1996), osmolality increased to the highest known levels for terrestrial reptiles. Rainfall events have been shown to increase tortoise metabolic rates (Henen *et al*. 1998), body mass, total body water volumes, and rehydrate blood plasma (Peterson 1996). Preston (1960) described fluctuations of haemodilution and haemoconcentration in the plaice, frog and turtle *Chrysemys picta*, and have been attributed to seasonality and more importantly, temperature.

Haematology can directly reflect the physiological condition of the animal, and has thus been used as an important diagnostic tool. The haematological effects of anaemia (Reavill 1994; Campbell 2004), malnutrition (Tavares-Dias *et al*. 2009), disease, chronic (Frye 1991) and parasitic infections (Knotkova *et al.* 2005) have been studied among reptiles and can readily be identified in the haemogram.

1.3 BLOOD PARASITES

In addition to describing blood cell morphology and counts, the complete haemogram involves the identification of blood parasites that may be present in the blood smear. Reptilian erythrocytes play host to a variety of protists, prokaryotic and viral infections, all occurring at various stages of the parasite's life cycle (Davies & Johnston 2000).

The Apicomplexa is well represented in Chelonia (Mihalca *et al*. 2008). Common examples include *Plasmodium* spp., a common genus of intracellular parasites in the blood of reptiles. Coccidian parasites, especially haemogregarines such as *Hepatozoon* spp., *Haemogregarina* spp. are intracellular blood parasites typical to chelonians (Lainson & Naiff 1998), and have been described in South African *Testunids* (Cook *et al*. 2009). In addition, haemoflagellates such as *Trypanosoma* spp. infect a wide range of reptiles. Many of the blood parasites require blood-sucking vectors for transmission, and ticks are common ecto-parasites to almost all tortoise species.

1.4 GEOMETRIC TORTOISES AND STUDY OBJECTIVES

Southern Africa has five genera of Testunids, or land tortoises (*Chersina*, *Homopus, Kinixys, Geochelone* and *Psammobates*) and 11 endemic species (Boycott & Bourquin 2000). South Africa has the richest tortoise diversity of any country, having five genera and 13 species, with seven being endemic to South Africa. Four genera and eight species of tortoise are found in the Northern and Western Cape Provinces alone (Branch 1998; Boycott & Bourquin 2000). The genus *Psammobates* comprises three species: *P. geometricus*, *P. oculiferus*, and *P. tentorius* (Boycott & Bourquin 2000). *Psammobates geometricus* (Linneaus, 1758), the geometric tortoise, has a limited distribution and is found only in the southwestern region of the Western Cape Province, (Boycott & Bourquin 2000). At present, three regions support geometric

tortoise populations, the Southwestern Coastal Lowlands, Worcester-Tulbagh Valley, and Ceres Valley. These populations are isolated from one another by natural barriers such as mountain ranges (Baard & Mouton 1993).

Psammobates geometricus is endemic to the vegetation type known as renosterveld. West coast renosterveld forms part of the Cape Floristic Kingdom, and experiences a typical Mediterranean climate (hot and dry summers followed by cool and wet winters). This renosterveld is characterised by mid-dense to closed and small-leaved evergreen shrubs of medium height, with regular clumps of broad-leaved, tall shrubs (Low & Rebelo 1996). Preferring well-drained, relatively open renosterveld areas of medium height and within 350 - 600 mm winter rainfall regimes, this close association of *P. geometricus* to renosterveld may be due to the availability of specific food plants, as geometric tortoises may have a specialized diet (Baard 1995; Boycott & Bourquin 2000). Its winter diet consists of annual grasses, geophytes and herbaceous taxa. In summer, it prefers perennial grasses, shrub and succulent components (Baard 1995). Currently, approximately 3% of the original renosterveld remains (Kemper *et al.* 2000), with the largest areas at the Elandsberg Nature Reserve and Tygerberg Hills. Renosterveld grows on soils highly suitable for cultivation and the conversion of renosterveld to agriculture is believed to be the major factor for the decline in geometric tortoises (Baard 1993).

As a result of such habitat and food specificity, *Psammobates geometricus* now appears in Appendix 1 on CITES, the South African Red Data Book of reptiles and amphibians (Baard 1993), and has moved from being listed as Vulnerable in 1982 to Endangered in the IUCN Red Data Book (IUCN 2011). In an attempt to protect the declining populations of this species, several nature reserves were established for this purpose in the southwestern Cape between 1971 and 1986 (Baard 1991). Despite increased legal protection of the geometric tortoise, their distribution is diminishing and has been attributed to many factors including increased predator pressure, with habitat destruction (through agriculture, spread of alien vegetation or uncontrolled burns) being the major cause of reduced numbers (Baard 1993). The continued decline of the species and threat to its environment resulted in the species being elevated to Critically Endangered in the most recent assessment (Baard & Hofmeyr, in press).

Physiological research on *P. geometricus* has been minimal, whilst this knowledge could prove critical to any conservation practice. In the wild, blood profiles provide a

minimally invasive tool that can support health evaluations, and baseline reference values are critical to establish the health of animals (Dessauer 1970; Frye 1991; Campbell 2004; Sykes & Klaphake 2008). The study objective of this investigation is to evaluate the effects of season and cohort on haematological values of wild geometric tortoise individuals under natural conditions to better understand their physiological responses to seasonal environmental fluctuations in the largest known remaining population.

2 HAEMATOLOGY

2.1 INTRODUCTION

The health status of reptiles is typically assessed through a physical examination and an evaluation of haematological and blood chemical values, which are compared to reference intervals, representing baseline values of healthy individuals (Jacobson 2007). Such baseline health values are presently lacking for most South African chelonian species. In reptiles, these reference intervals show much variation between and within species, with many factors – both intrinsic and extrinsic – making it difficult to establish reference blood intervals for any species. Sex, age, and nutritional status of individuals are important intrinsic factors (Mader 2000). Some extrinsic factors are seasonal effects, including temperature changes, hydration state and diet availability (Jacobson 2007). Habitat, captivity and method of blood sample collection may also influence haematological values (Lopez-Olvera *et al.* 2003; Jacobson 2007). Deviations from expected values for healthy individuals can be used to assess the impact of stresses such as habitat loss (Brenner *et al*. 2002), drought (Christopher *et al.* 1999) and infectious diseases (Tavares-Dias *et al.* 2009) on wild tortoise and turtle populations.

Due to its easily diagnostic application, chelonian haematological research has increased in recent years, with most studies being undertaken on American, European and Asian turtle and tortoise species (Bolten & Bjorndal 1992 ; Knotkova *et al*. 2002; Perpinan *et al*. 2008). Results of such studies report that blood values change with an individual's age and sex. For most species, males show higher packed cell volumes, haemoglobin concentrations and/or red cell counts (Frair 1977). This condition - common among vertebrates (Gardner & Gorshein 1973) - is attributed to the erythropoiesis-stimulating effects of testosterone and other androgenic steroids (Zitzmann & Nieschlag 2004). In a long-term study of green iguanas, Knotek *et al*. (2006) showed that as age increases, packed cell volume and red cell counts decrease as haemoglobin concentrations and computed red cell indices increase. Casal & Oros (2007) noted significant differences between adult and juvenile red blood cell count and packed cell volume in green turtles. Whilst this pattern may be typical, studies with small sample sizes reported no differences in blood values between males and females, or adults and juveniles (Bolten & Bjorndal 1992; Martinez-Silvestre *et al.* 2001), which highlight the importance of sample size in quantitative study design.

In addition to age and sex, environmental conditions are known to affect chelonian blood values, owing to their ectothermic nature (Reavill 1994; Mader 2000). As warmer temperatures facilitate an increase in ectotherm activity, metabolic changes are required to accommodate the associated raised energy demands (Kuchling 1999). This can be measured by changes in the haemogram, since erythrocytes function primarily in oxygen delivery (Hartman & Lessler, 1964). As blood plasma volume is a function of the animal's hydration state, rainfall patterns are also closely associated to changes in blood profiles. In addition to reproductive cycle, Christopher *et al*. (1999) attributed most haematological variations to the availability of food and water in the Desert tortoise, *Gopherus agassizii*. In a further study on *G. agassizii*, Dickinson *et al*. (2002) found that rainfall accounted for most variation in seasonal and annual haematological values. Surveying the literature on chelonian haematology, Frair (1977) noted that for Chelonia in general, packed cell volume and red cell count are higher in winter than summer, which he attributed to the seasonality associated with erythropoiesis. This is echoed by Hidalgo-Vila *et al*. (2007), who proposed that wide mean cell volume ranges in Mediterranean pond turtles (*Mauremys leprosa*) could be explained by seasonal differences in erythrocyte volume, whereas in a comprehensive study on packed cell volumes of *G. agassizii*, Peterson (2002) attributed the seasonal PCV fluctuations to what he termed the hydration hypothesis.

To my knowledge, the only previous study to present blood values on *P. geometricus* was in 1938 – a cytological study on *Testudo geometrica* (Bernstein) and no previous study has attempted to assess the possible effects of age, gender, or season thereupon. The aim of this investigation was to establish baseline reference haematological values for males, females and juveniles of this Critically Endangered species and to determine how these cohort values change with seasonal fluctuations in environmental conditions.

2.2 MATERIALS AND METHODS

2.2.1 Sampling procedure

Blood samples were obtained over four seasons at Elandsberg Nature Reserve (3 800 ha; 33° 26' S; 19° 01' E) in the southwestern Cape, South Africa, from 26 to 42 healthy, free-ranging geometric tortoises (including males, females and juveniles) per

season (Table 2.1). Weather data for the study period were obtained from AgroMet-ISCW for the nearest weather station, De Hoek (33.15° S, 19.03° E), 25 km north of Elandsberg, with a similar orientation to the Elandsberg mountain range.

Table 2.1 Field trip dates and sample sizes of female, male and juvenile geometric tortoises at Elandsberg Nature Reserve, South Africa.

Season	Dates (2000-2001)	Female	Male	Juvenile	Total
Spring	30 August - 16 September	15	11	5	31
Summer	11-19 December	11	8	7	26
Autumn	2-10 April	10	12	5	27
Winter	19-27 June	16	13	13	42

In the field, I weighed individuals to the nearest 0.1 g with an Ohaus digital balance and used vernier callipers to record body parameters to the nearest 0.1 mm. I recorded: straight carapace length (SCL) from the nuchal to the supracaudal scute, shell width (SW) across marginal scutes six to seven, and shell height (SH) over the third vertebral scute. I used these measurements to calculate shell volume (SV, cm^3) from a modified formula for an ellipsoid as derived by Loehr *et al.* (2004): $SV = \pi * SCL * SH * SW / 6000$. Body mass (BM) fluctuations can be used to assess changes in body condition (Hailey 2000), but BM is often scaled to body size to allow direct comparisons among individuals of different sizes (Jakob *et al.* 1996). I thus calculated a body condition index (BCI-ratio) as BM / SV, as described by Loehr *et al.* (2007). This index uses a three-dimensional measure of body size as opposed to a one-dimensional measure such as SCL, as is commonly used (Jacobson *et al.* 1993). Additionally, I calculated regression equations for mass-log_{10} on SV-log_{10} for each cohort in order to use residuals as an indicator of body condition (BCI-residuals; Green 2001). Males, females and juveniles were distinguished by external morphology (Fig. 2.1), as described by Baard (1990). Before release at its place of capture, each tortoise received a unique number by filing shallow notches in specific marginal scutes, as in Honegger (1979).

I sampled blood from unanaesthetised tortoises immediately after capture to limit stress-induced changes to blood parameters. The mass of the animals determined the maximum blood volume sampled and I took care not to exceed 0.5% of the animal's field body mass (a conservative veterinary standard). I used a 25 G needle with a 1 or 2 ml syringe to collect blood from either the jugular vein or carotid artery

(Fig. 2.2). Sampling normally took 1 or 2 minutes and I aborted attempts if an adequate sample has not been obtained in approximately 5 minutes. The animals were kept under observation for 24 hours and during the dry season, I provided access to drinking water before returning the animals to the capture site.

Figure 2.1 External morphology of *Psammobates geometricus* cohorts: a) female, b) male and c) juvenile.

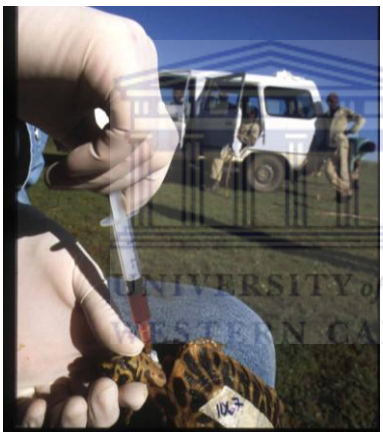

Figure 2.2 Blood sampling from the jugular vein of *Psammobates geometricus*.

After blood sampling, a 0.2 ml aliquant of whole blood was kept on ice for haematological analyses, which were completed within 24 hours of sampling. Two heparinised microhaematocrit tubes were filled with blood and centrifuged for 10 minutes at 7000 rpm to determine packed cell volume (PCV). Red blood cells were counted (RCC) in duplicate with a haemocytometer, and haemoglobin concentration (Hb) was measured with a BMS haemoglobinometer. I used dual cell analysis for haemoglobin when concentrations were less than 4 g/dL. A correction factor of 0.58 (determined experimentally) was then used to correct dual cell values.

I calculated red blood cell indices from standard formulae using RCC, PCV and Hb values by the method in Duncan *et al.* (1994) as follows:
Mean cell volume (MVC) (femtolitres) = (PCV x 10) / RCC (millions);

Mean cell haemoglobin (MCH) (picograms) = (Hb x 10) / RCC (millions);
Mean cell haemoglobin concentration (MCHC) (g / dL) = (Hb x 100) / PCV.

2.2.2 Statistical analysis

I used SigmaStat (SPSS Inc., Chicago, U.S.A. version 2.03) to test each physical measurement and haematological parameter for normality and equal variance to ascertain if the data satisfied the requirements for parametric tests. Mean and standard deviation were calculated for parametric data. I used paired t-tests to compare monthly rainfall and temperature data for 2000 and 2001. To test for the effects of season, cohort and a possible interaction of the two factors, I performed two-way analysis of variance (ANOVA) on morphometric measurements, BCI's, and the six haematological parameters. Student-Newman-Keuls tests were used as multiple post-hoc comparisons to identify specific differences between groups. Data for SCL and SV failed normality and/or equal variance. In these instances, I still presented two-way ANOVA results after cross-checking if results were similar when using multiple one-way ANOVAs with sequential Bonferroni corrections. I used linear regressions to assess the possible effects of body condition (BCI-ratios) on blood parameters within cohorts and within seasons. All differences were considered statistically significant at values of $P \leq 0.05$.

2.3 RESULTS

2.3.1 Weather conditions

Typical of the mediterranean-type climate experienced at the study site, most rain fell from late autumn to early spring, with annual rainfall being more than one and a half times higher in 2001 than in 2000 (Fig. 2.3a). Yet, rainfall did not differ significantly between the two years (t_{11} = 1.87, P = 0.089). Similarly, minimum and maximum temperatures between the years did not differ (P > 0.19; Fig. 2.3b). During the spring sampling period, September 2000, rainfall was high (117 mm) and temperatures were mild (T_{max} and T_{min} = 20.2 and 9.1°C). Subsequently, rainfall decreased and temperatures increased toward summer sampling in December (rain=14 mm; T_{max} and T_{min} = 30.4 and 15.5°C). Rainfall remained low and the first substantial autumn rains fell during the first week of autumn sampling (April rainfall = 33 mm). Temperatures during autumn sampling were higher than during spring, but lower than during summer (T_{max} and T_{min} = 25.3 and 14.9°C). Winter sampling was preceded by high rainfall in May with drier conditions (41 mm) and lower temperatures (T_{max} and T_{min} = 18.8 and 8.0°C) in June.

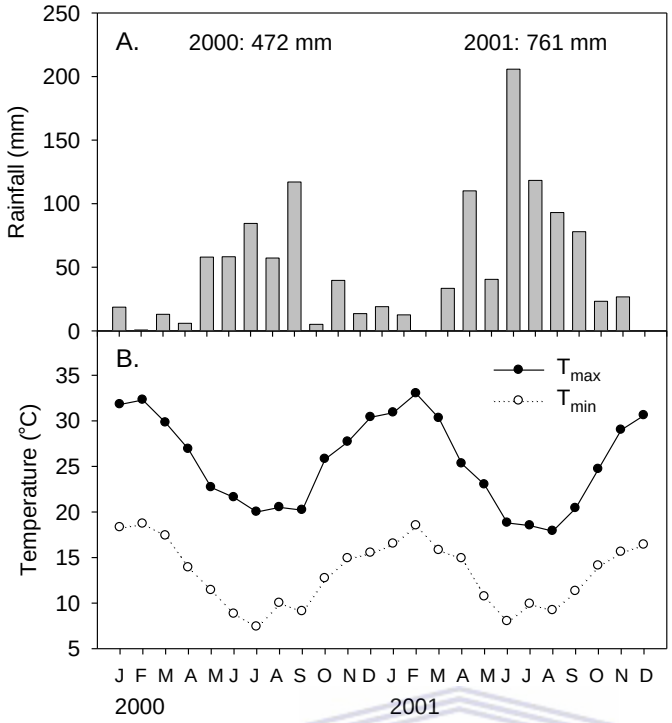

Figure 2.3 Weather data captured at De Hoek weather station showing (a) monthly rainfall (mm) and (b) maximum and minimum temperatures (°C) in 2000 and 2001. Annual rainfall for each year is also indicated.

2.3.2 Body size and condition indices

Psammobates geometricus showed sexual size dimorphism with females being larger than males, and both sexes being larger than juveniles (Fig. 2.4). These size differences were valid for SCL ($F_{2,113}$ = 367, P < 0.001; Fig. 2.4a), SV ($F_{2,113}$ = 325, P < 0.001; Fig. 2.4b) and BM ($F_{2,114}$ = 352, P < 0.001; Fig. 2.4c). Mean body size of the cohorts did not differ among season with respect to SCL or SV (both P > 0.49; Table 2.2). While there was an interaction of cohort and season for SCL ($F_{6,113}$ = 2.48, P = 0.027), but not for SV (P = 0.09), the within cohort post hoc comparisons for SCL were not significant. Body mass changed with season ($F_{3,114}$ = 5.75, P = 0.001; Table 2.2), with an interaction between cohort and season ($F_{6,114}$ = 2.50, P = 0.026). Overall, the tortoises weighed heavier in winter, spring and summer than in autumn, but within cohorts, seasonal mass changes were significant for females only and not for males and juveniles. Winter, spring and summer masses of females were higher than in autumn, and their winter mass exceeded also their summer mass.

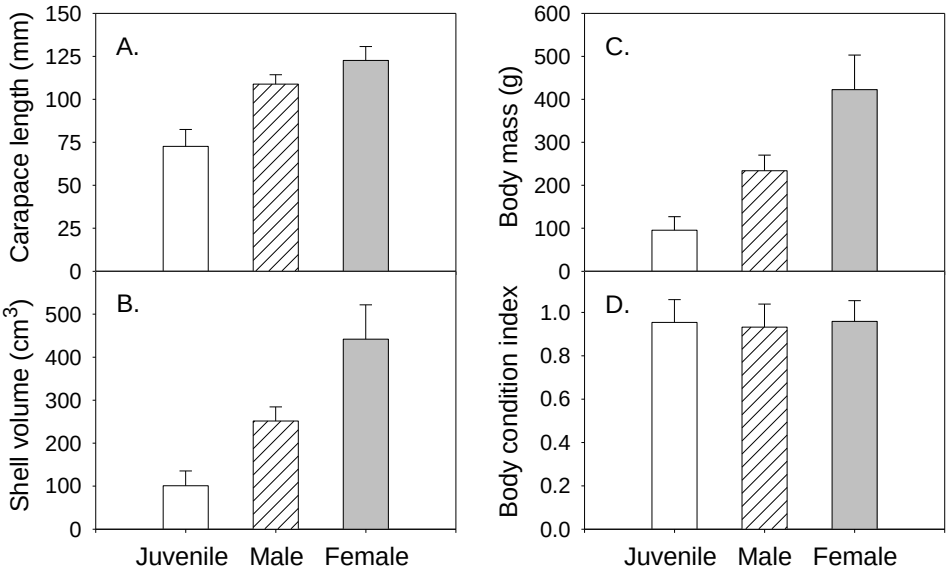

Figure 2.4 Body size as carapace length (a), shell volume (b) and body mass (c), and condition index (d) based on ratios (mean ± SD) of *Psammobates geometricus* cohorts measured at the Elandsberg Nature Reserve from 2000 to 2001. Sample sizes for juveniles, males and females, respectively, were 30, 44 and 51, except females had 52 samples for mass.

The BCI ratios of geometric tortoises were not affected by cohort (P = 0.303; Fig. 2.4d), but showed a strong seasonal change ($F_{3,113}$ = 60.8, P < 0.001; Table 2.2). The interaction between the two factors just failed significance ($F_{6,113}$ = 2.17, P = 0.051). Similar to body mass changes, BCI's were higher in winter, spring and summer than in autumn. However, in contrast to body mass results, the seasonal pattern for BCI applied to females, males and juveniles. Regressions of mass-$\log_{10}$ on SV-$\log_{10}$ for geometric tortoise cohorts did not always meet parametric assumptions but the BCI-residuals nevertheless gave similar results to BCI's based on ratios (cohort: P = 0.22, season: $F_{3,113}$ = 55.7, P < 0.001, interaction: P = 0.40).

Table 2.2 Body size and condition of juvenile (J), male (M) and female (F) *Psammobates geometricus* expressed as means, standard deviation and sample sizes (n) for straight carapace length (SCL), shell volume (SV), body mass, and body condition index (BCI) during the four seasons of the study from 2000 to 2001.

		Spring	Summer	Autumn	Winter
SCL (mm)	J	66.1 ± 12.3 (5)	75.2 ± 7.7 (7)	76.2 ± 12.8 (5)	72.3 ± 8.4 (13)
SCL (mm)	M	108.5 ± 3.1 (11)	106.8 ± 5.2 (8)	111.4 ± 6.5 (12)	108.1 ± 5.7 (13)
SCL (mm)	F	124.9 ± 4.8 (15)	118.0 ± 10.4 (10)	119.4 ± 10.9 (10)	125.4 ± 5.3 (16)
SV (cm^3)	J	75.8 ± 33.7 (5)	110.6 ± 31.2 (7)	116.2 ± 47.6 (5)	99.6 ± 28.9 (13)
SV (cm^3)	M	254.4 ± 19.9 (11)	236.7 ± 29.6 (8)	266.9 ± 35.4 (12)	244.4 ± 38.1 (13)
SV (cm^3)	F	457.5 ± 58.9 (15)	404.8 ± 96.7 (10)	415.4 ± 109.7 (10)	467.5 ± 52.5 (16)
Mass (g)	J	76.2 ± 33.8 (5)	105.7 ± 32.6 (7)	86.3 ± 29.1 (5)	100.8 ± 30.4 (13)
Mass (g)	M	246.2 ± 24.1 (11)	239.1 ± 34.1 (8)	210.0 ± 41.9 (12)	242.1 ± 34.5 (13)
Mass (g)	F	437.4 ± 64.7 (15)	412.5 ± 91.5 (11)	344.4 ± 81.6 (10)	464.6 ± 47.3 (16)
BCI (g cm^{-3})	J	1.00 ± 0.04 (5)	0.95 ± 0.05 (7)	0.76 ± 0.08 (5)	1.01 ± 0.05 (13)
BCI (g cm^{-3})	M	0.97 ± 0.04 (11)	1.01 ± 0.03 (8)	0.78 ± 0.08 (12)	0.99 ± 0.04 (13)
BCI (g cm^{-3})	F	0.96 ± 0.08 (15)	1.02 ± 0.04 (10)	0.84 ± 0.09 (10)	1.00 ± 0.06 (16)

2.3.3 Effect of body condition on blood values

Regression analysis showed that body condition influenced the PCV ($F_{1,120}$ = 4.05, P = 0.046, r^2 = 0.033) and RCC ($F_{1,121}$ = 6.03, P = 0.015, r^2 = 0.047) of geometric tortoises, but r^2 values were low. Within cohorts, regressions were significant only for male RCC ($F_{1,42}$ = 5.54, P = 0.023, r^2 = 0.117), and within seasons, regressions were significant only for RCC in autumn ($F_{1,25}$ = 8.62, P = 0.007, r^2 = 0.256).

2.3.4 Effects of cohort and season on blood values

Packed cell volumes ranged from 13.9% to 27.1% for juveniles, 15.4% to 32.1% for males, and 16.6 to 27.6% for females. Packed cell volumes below 20% were recorded in all seasons, although the lowest values were recorded in autumn and winter. Both cohort ($F_{2,111}$ = 13.48, P < 0.001) and season ($F_{3,111}$ = 2.72, P = 0.048) influenced the PCV of geometric tortoises (Fig. 2.5a), but there was no interaction between cohort and season (P = 0.29). Males had a higher PCV than females and juveniles had, while female and juvenile PCVs did not differ. Post hoc tests showed

no difference among seasons. When I forced interaction effects for the ANOVA, the differences among cohorts were limited to summer and autumn, and male PCVs were higher in summer and autumn than in winter.

Haemoglobin concentrations ranged from 4.2 to 7.7 g/dL for juveniles, 3.5 to 9.7 g/dL for males, and 5.2 to 9.0 g/dL for females. In spring, Hb of only two juveniles were sampled. Haemoglobin concentration showed cohort ($F_{2,109}$ = 11.94, P < 0.001) and seasonal ($F_{3,109}$ = 8.39, P < 0.001) differences with no interaction between the two (P = 0.39) (Fig. 2.5b). Male Hb was highest and female Hb higher than that of juveniles, with Hb of cohorts combined being lowest in winter. Within-group analyses (forced interaction) revealed that the overall seasonal trend applied to males only, with females having higher Hb values only in summer and spring than in winter, and juveniles showing no seasonal Hb differences. Furthermore, there were no cohort differences in spring and winter, whereas male Hb concentrations in summer and autumn were highest, with no difference between female and juvenile concentrations.

Red blood cell counts ranged from 0.29 to 0.70 million/uL in juveniles, 0.32 to 0.89 million/uL in males, and 0.42 to 0.81 million/uL in females. Cohort influenced RCC ($F_{2,112}$ = 9.16, P < 0.001). Overall, male RCC was highest, with female and juvenile RCCs being similar. The RCC of geometric tortoises just failed significance for season ($F_{3,112}$ = 2.41, P = 0.071) (Fig. 2.5c); summer RCC tended to be higher than spring RCC. Similarly, an interaction effect just failed significance ($F_{6,112}$ = 1.98, P = 0.075); males had higher RCCs than females and juveniles only in summer and autumn, and male RCCs were higher in summer and autumn than in spring and winter.

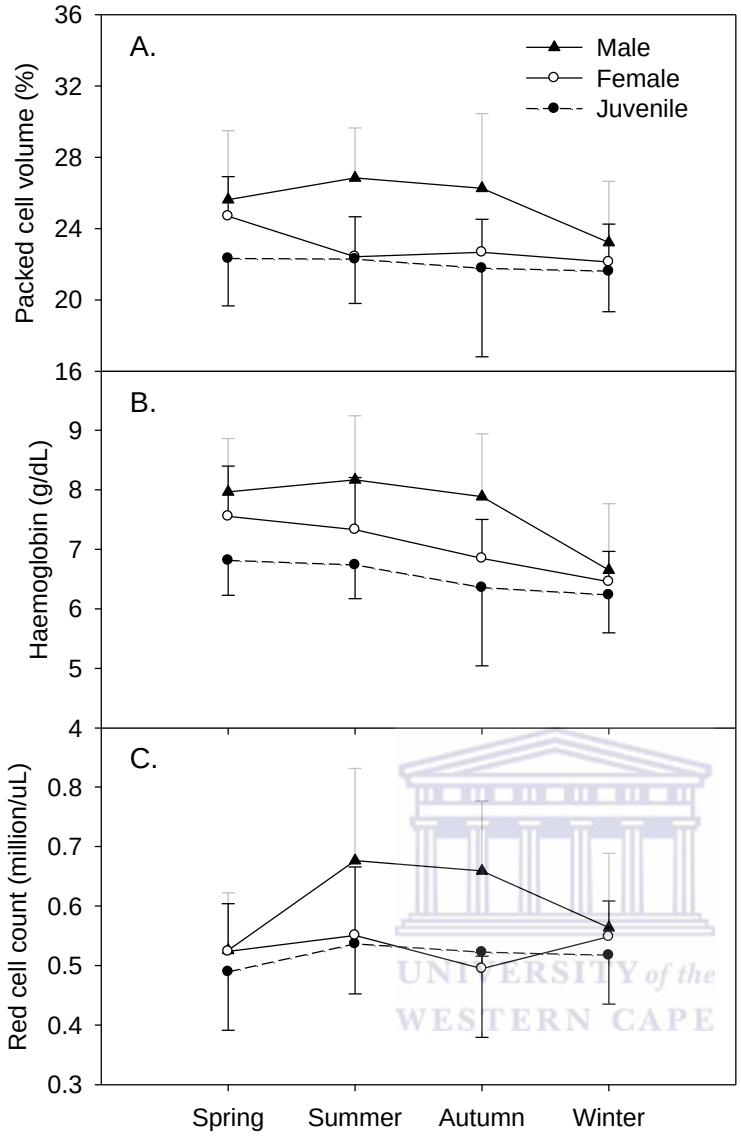

Figure 2.5 Seasonal values (mean ± SD) for packed cell volume (a), haemoglobin concentration (b) and red blood cell count (c) of *Psammobates geometricus* juveniles, males and females at Elandsberg Nature Reserve from August 2000 to June 2001.

2.3.5 Effect of cohort and season on RBC indices

Mean cell volume ranged from 341.9 to 547.9 fL in juveniles, 313.6 to 651.3 fL in males, and 322.9 to 580.8 fL in females. Mean cell volume did not differ among cohort ($P = 0.85$), but changed seasonally ($F_{3,110} = 8.80$, $P < 0.001$; Fig 2.6a) with no interaction of cohort and season ($P = 0.27$). Overall, geometric tortoise MCV was highest in spring with no differences among the other seasons. Within-cohort evaluation (forced interaction) showed that the seasonal pattern applied only to

males, whereas spring MCV of females exceeded only winter and summer values, and MCV of juveniles did not differ among season.

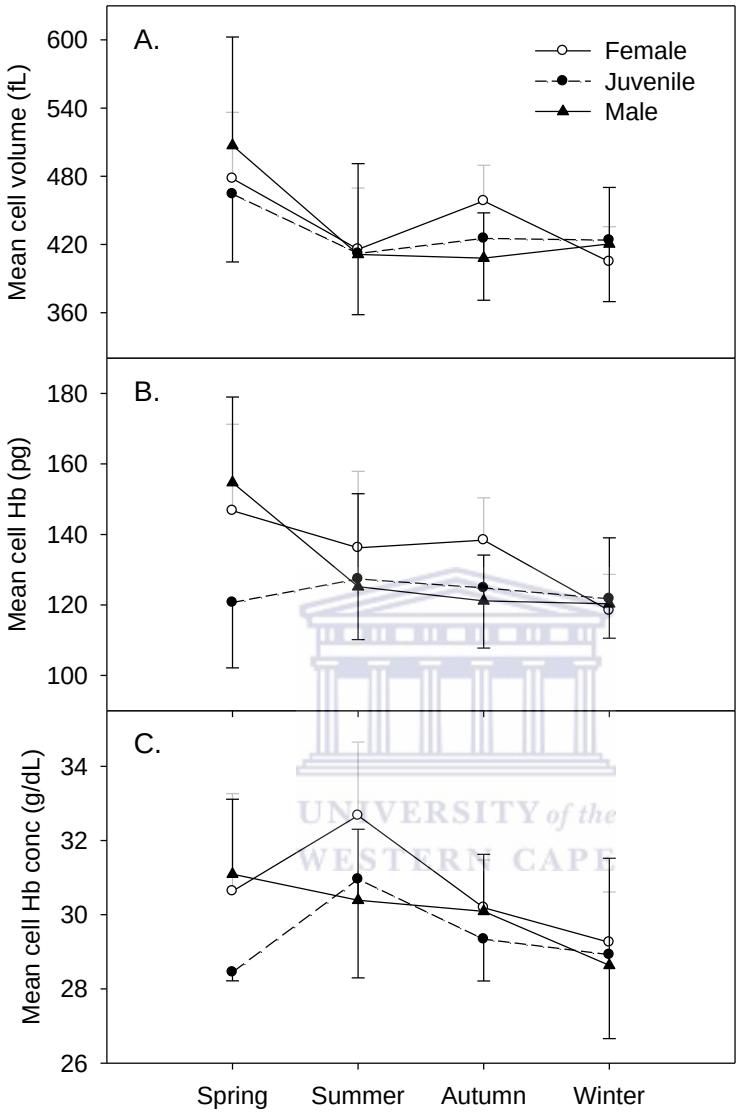

Figure 2.6 Seasonal changes (mean ± SD) for mean cell volume (a), mean cell haemoglobin (b) and mean cell haemoglobin concentration (c), of *Psammobates geometricus* juveniles, males and females at Elandsberg Nature Reserve from August 2000 to June 2001.

Mean cell haemoglobin ranged from 98.9 to 150.0 pg in juveniles, 89.8 to 201.3 pg in males, and 97.3 to 206.3 pg in females. Mean cell haemoglobin (Fig. 2.6b) showed a seasonal response ($F_{3,109}$ = 4.64, P = 0.004) and was not affected by cohort (P = 0.089), with no interaction between the two factors (P = 0.084). For combined cohorts, spring MCH values exceeded those in winter but not those in autumn or

summer. By forcing interactions, the results indicated that male MCH was higher in spring than in all other seasons, while for females it was lowest in winter, and juveniles showed no seasonal change. Furthermore, the MCH of males exceeded that of juveniles in spring.

Mean cell haemoglobin concentration ranged from 25.85 to 35.12 g/dL in juveniles, 22.9 to 34.0 g/dL in males, and 25.83 to 35.84 g/dL in females. In geometric tortoises, MCHC showed seasonal changes ($F_{3,107}$ = 6.65, P < 0.001; Fig. 2.6c), just failed significance for cohort (P = 0.077), and showed no interaction between the two factors (P = 0.36). Overall, summer MCHC was higher than winter and autumn. Within-cohort evaluation (forced interaction) indicated that for females, MCHC was highest in summer, for males, spring MCHC was higher than winter values, whilst juveniles showed no seasonal change in MCHC.

2.4 DISCUSSION

2.4.1 Effect of season

Ectotherms are defined as being dependent on external heat sources for thermoregulation, in contrast to endotherms (mammals and birds), which can adjust their body temperatures by altering metabolic rates. Reptilian physiological and behavioural mechanisms regulate activities within their preferred optimum temperature range, and in temperate climates, metabolic activities may fluctuate with seasonal variations (Kuchling 1999). Thermoregulation is an important driving factor upon ectothermal physiological processes, as noted in many species that practice hibernation, aestivation or brumation (Jacobson 2007).

There is evidence that *P. geometricus* adapts its blood physiology in response to environmental conditions, as seasonality showed significant effects on most blood parameters tested in this study. During the cold winter months, lowest haemoglobin concentration and mean cell haemoglobin concentrations were observed, implying a reduction in oxygen transport which is indicative of slower metabolic rates (Jacobson 2007). In concordance with this trend, geometric tortoise mean cell haemoglobin concentrations were higher in the warmer months of summer and spring. Following winter rainfall, and the resulting abundance of food taxa (Balsamo *et al*. 2004), elevated MCHC in spring and summer suggests an erythropoietically-enabled rise in metabolic activity, in order to maximise increased foraging opportunities.

In conjunction to the effects of varying temperature ranges, seasonality also brings about a change in water availability. Seasonal hydration fluctuations can be observed through variations in packed cell volume, as plasma volume is influenced by hydration state. Significant correlations between packed cell volume and level of dehydration and malnourishment are reported for tortoises (Christopher *et al*. 1999) and turtles (Tavares-Dias *et al*. 2009). Geometric tortoise PCV, however, showed little evidence of dehydration in the dry season, This is typical in certain arid or semi-arid tortoises, which have evolved anatomical and physiological adaptations enabling them to thrive under water-restricted conditions. Notably, Jacobson (2007) reports that tortoises have proportionally, the largest urinary bladder, which serves as a storage site for water and in which osmoregulatory ions are concentrated during periods of drought. This anhomeostasis of body plasma is observed and described in the Desert tortoise (*G. agassizii*, Peterson 1996; Peterson 2002). Since packed cell volumes are also affected by red cell count and size, it is difficult to identify responses to isolated factors. Rehydration after rains in autumn may cause a haemodilution – resulting in lower PCV – however, there is little evidence for this in *P. geometricus*, but this may be found to be the case in the few individuals sampled shortly after the rains.

Whilst rainfall itself may not bear a direct effect on blood values, it does influence the availability of herbaceous food plants (Joshua *et al.* 2005), and hence nutritional states – or condition of tortoises. This can be observed by seasonal changes of geometric tortoise body mass, being lowest in autumn, the driest sampling period. It is interesting to note that once mass was scaled to size (body condition), the same pattern applied to each cohort. Assessing body condition in greek tortoises (*Testudo* spp.) among different sites, Willemsen & Hailey (2002) report that while differences in body condition between sites in spring were related to activity and thermoregulation, differences in summer and autumn were related to food availability. Hailey (2000) advises that seasonal variation in condition index for males, females and juveniles be interpreted in relation to activity of these cohorts, including reproductive requirements.

2.4.2 Effect of cohort

The reproductive state of tortoises is an important intrinsic factor on blood physiology, with timing of seasonal reproduction being influenced by external cues (such as temperature or water availability; Kuchling 1999). Geometric tortoise mating season has been observed to occur in the summer months (Hofmeyr & Henen,

unpublished data). In this study, the most pronounced seasonal blood value differences occured in summer and autumn in males. Packed cell volume, red blood cell count and haemoglobin concentration were highest in males in these dry months, with no significant differences between females and juveniles. The higher male haematological values are common in vertebrates, and while research has been undertaken on mammalian species, the mechanisms apply in reptiles also. The erythropoietic-stimulating effects of androgens (Gardner & Gorshein 1973) has been ascribed to the stimultion of erythropoietin, promotion of erythroid formation in bone marrow, enhancement of iron incorporation in red blood cells and the enhancement of haemoglobin synthesis (Zitzmann & Nieschlag 2004). The increased PCV, Hb and RCC in males in these summer months, could be attributed to increased androgenic hormones during the mating period. This suggests male erythropoiesis occurs to accommodate raised metabolic demands of mate-seeking and male aggression during the mating period.

Seasonal blood values in female geometric tortioses, however, follow a different pattern. Female haemoglobin concentrations were high in spring and summer, whilst mean cell haemoglobin concentration was highest in the summer months. Mean cell volume was high in spring, and in contrast to males, appears to rise again in autumn. Van Bloemestein (2005) noted that geometric tortoise females in spring were more active than males, moving more often and more randomly, and related this movement pattern to foraging activity. It is likely that erythropoiesis in female geometric tortoises is necessary to facilitate the extra metabolic requirements of foraging activities to satisfy higher nutritional demands associated with larger body size and egg production. Geometric tortoises mate in the summer and females nest from late winter to early summer (Hofmeyr *et al.* 2006). A new vitellogenic cycle starts in spring with rapid enlargement of follicles in autumn, in preparation for ovulation after the first autumn rains (Hofmeyr & Henen, unpublished data). The increased MCV in autumn suggests that females may experience a second phase of erythropoiesis to accommodate their reproductive requirements.

The blood values of juvenile geometric tortoises showed no significant seasonal effect which appears to be typical for juveniles in other chelonians (Anderson *et al*. 1997). Nevertheless, several studies indicated that blood values of juveniles differ from that of adults. In a long-term study of green iguanas, Knotek *et al*. (2006) showed that with aging, packed cell volume and red cell counts decrease, as haemoglobin concentrations and computed red cell indices increase. Casal & Oros

(2007) found also that adult green turtles displayed lower packed cell volumes than juveniles, while in this species, adults had higher red cell counts than those of juveniles. Differences between adults and juveniles could be attributed to reduced energy requirements associated with smaller body size, as well as the absence of reproductive stresses in juveniles (Kuchling 1999; Jacobson 2007). For juvenile geometric tortoises, only their Hb concentration was lower than that of males and females. Juvenile PCV and RBCC did not differ from female values, and their RBC indices did not differ from either adult sex. The close correspondence of juvenile blood values with adults, may reflect species specific responses to environmental conditions, or it may be a consequence of low juvenile sample sizes, particularly in spring.

Geometric tortoise haematology appears to follow similar patterns to those reported for other studied tortoises. Higher male PCV, Hb and RCC are recorded for land tortoises *Gopherus agassizii*, *Kinixys erosa*, *Testudo graeca* and *T. hermanii* (Oyewale *et al*. 1998; Christopher *et al*. 1999; Peterson 2002) as well as aquatic turtle species (Anderson *et al*. 1997; Hidalgo-Vila *et al*. 2007). Krasilnikov (1963, as quoted in Frair ,1977) reports that seasonal variations in both PCV and RCC may result from fluctuations in erythropoiesis, quoting a study of 18 reptilian species that included tortoises, where it was found that the peak of erythropoiesis occur in summer with a lesser spring peak after hibernation when feeding began.

Psammobates geometricus haematological values fall within ranges described for other tortoise species. Red blood cell values for *Kinixys erosa*, *Gopherus agassizii* (Oyewale *et al.* 1998), and *Testudo marginata* (Martinez-Silvestre *et al*. 2001), respectively, include average PCV of 30, 29 and 23%; Hb of 10, 9.7 and 6.2 g/dL; and RCC of 0.7, 0.5 and 0.6 million/uL. This suggests a relatively healthy *P. geometricus* population, as low PCV, Hb and RCC values could be indicative of malnourishment, dehydration or sickness (Frair 1977; Christopher *et al*. 1999; Tavares-Dias *et al*. 2008). Baseline studies of natural wild populations have proven to be important in assessing the physiological state of individuals, which is particularly useful in captive or farming programs (Willemsen *et al*. 2002; Knotkova *et al*. 2005).

2.5 CONCLUSIONS

Haematological evaluations have been used widely as successful tools in determining health status of a variety of chelonian species. A range of external conditions (temperature, seasonal patterns, rainfall) and internal factors (age, sex, reproductive state) influence tortoise physiology, and such associated changes are reflected in the haemogram.

The haematology of geometric tortoises changed with season, and environmental fluctuations explained these changes only partially. The physiological responses of males, females and juveniles differed substantially and can be related to reproductive needs. Thus, baseline health studies should assess cohorts separately, and seasonal variations should be considered.

To ensure that baseline value ranges are truly meaningful, researchers should maintain standardardised methods of laboratory and analytical technique, as well as consider the important role of the environmental and individual circumstances on blood parameters. This is made difficult by the plasticity of chelonian physiological strategies and intricate complexity of environmental influences thereupon.

3 RED BLOOD CELL HISTOLOGY

3.1 INTRODUCTION

A thorough haematological evaluation includes an assessment of red and white blood cell counts and morphology (Campbell 2004). The sampling of blood to make blood smears is minimally invasive and easily performed in wild populations. Blood smears have been used with success to indicate an animal's health, and can reflect the state of physiological processes (Arikan & Cicek 2010). In the clinical application of reptilian histological examinations, the polychromatic Romanowsky stains have been used with success to identify the different cell lineages in haemopoietic tissues (Couture & Hafer 2004). Although histological techniques for reptiles are similar to those used for mammals, slight differences exist, due to nucleation of reptilian erythrocytes and pluripotency of thrombocytes (Pendl 2006). External environmental factors such as temperature, and internal factors that influence the activity level of individuals, e.g., hibernation or breeding status, have great effects on the physiology of ectotherms, which is reflected in the wide range of interspecific and intraspecific variation of reptilian blood cell morphology (Campbell 2004; Strik *et al*. 2007).

Wintrobe (1933, as cited in Arikan & Cicek 2010) suggested that the size of red blood cells depicts the place of a species along the evolutionary scale, with large, nucleated erythrocytes belonging to lower vertebrates while small, anucleated erythrocytes are found in the higher vertebrates. Based on this principle, results obtained from a study of the blood cell morphologies of Turkish herpetofauna (Arikan & Cicek 2010) place reptiles intermediate between amphibians and birds. Since the red blood cells transport oxygen and carbon dioxide throughout the body, the surface area to volume ratio of red blood cells is an important determining factor for gaseous exchange in the tissues. Smaller cells have a larger surface to volume ratio and are more efficient in gas exchange than larger cells. Likewise, elliptical cells provide a larger surface area for gaseous exchange than round cells (Hartman & Lessler 1964). Metin *et al*. (2008) report a positive correlation between erythrocyte size and nuclear size for testudinids, and Shadkhast *et al.* (2010) linked elongated nuclei of *Testudo horsfieldii* to an increased surface area for exchange with erythrocyte cytoplasm. Based on comparative studies of red blood cell sizes, Arikan & Cicek (2010) found that cell and nuclear shape and size of erythrocytes render terrestrial species more efficient at gaseous exchange than aquatic species.

Due to the clear functional role of the erythrocyte in metabolic facilitation, morphological descriptions, including shape, size, colour and irregularities of both erythrocytes and nuclei, are important characteristics and have been described for a few tortoise (Shadkhast *et al*. 2010) and turtle (Metin *et al*. 2008) species, although knowledge in this field is still lacking for many chelonians. Hartmann & Lessler (1964) describe reptile red blood cell size ranges of 15 to 19 µm in length, widths between 7.5 and 12 µm and nuclear dimensions between 5.1 and 8 µm. Among the reptiles studied, chelonians have the largest erythrocytes, and among chelonians, the smallest erythrocytes are observed in terrestrial species (Arikan & Cicek 2010).

The lifespan of red blood cells is not finite; senescent cells disintegrate and are replaced through erythropoiesis. In all animals, erythropoiesis occurs early during embryogenesis in the blood islands of yolk sacs, and while this is the main embryonic erythropoietic organ, as development continues, erythropoiesis is noted to occur also in the liver and spleen (Palis & Segel 1998). Vasse & Beaupain (1981) have observed differentiation stages towards mature erythrocytes in early somite stages of turtle embryos. After hatching, most erythropoiesis occurs in the leg bone marrow, whilst the liver remains erythropoietically functional in young turtles (Vasse & Beaupain 1981).

Terminology for the different developmental stages of erythrocytes is inconsistent. Pienaar (1962) described six morphologically identifiable stages in reptile erythroid differentiation; categorised into primitive (pro-erythroblasts, erythroblasts and basophilic normoblasts), immature (polychromatophilic normoblasts and pro-erythrocytes) and mature erythrocytes. Literature that is more recent generally refers to early stages in the continuum of erythrocyte development as different types of rubricytes (e.g., prorubricytes, basophilic rubricytes, polychromatophilic rubricytes and metarubricytes), and to the more advanced immature stages as polychromatophils or polychromatophilic erythrocytes (Bounous & Stedman 2000; Campbell 2004; Strik *et al*. 2007). The term polychromatophilic relates to the cytoplasm showing both basophilic and eosinophilic staining properties after haemoglobin production started.

Peripheral red blood composition always contains a percentage of immature red cell stages, mostly polychromatophilic erythrocytes and rubricytes, and particularly among juveniles (Pienaar 1962; Campbell 2004). It is generally accepted that immature erythrocytes change from a round to oval shape as they mature, have

intensely basophilic cytoplasm in the early stages, have large, dark-staining nuclei, and are smaller than mature erythrocytes in tortoises (Reavill 1994; Mader 2000; Zhang *et al.* 2011). Mature cells are easily identifiable as large, flattened, oval-ellipsoid cells with centrally positioned oval-ellipsoid nuclei (Reavill 1994; Campbell 2004; Zhang *et al.* 2011). Pienaar (1962) distinguished two genealogically different lineages of immature erythrocytes, namely lymphoid-type pro-erythrocytes and stem cell-type pro-erythrocytes, the former being the more common form. The younger rubricytes also appear in the two different varieties, although there are fewer in circulating blood. The two genealogically different forms of erythrocyte progenitors vary in nuclear and cytoplasmic staining quality and intensity, and undergo different changes as the cells mature. The lymphoid-type derivations are smaller cells and Frye (1991) as well as Pendl (2006) propose that these are rather thrombocyte derivations. The occurrence in low frequencies of immature erythrocytes in peripheral blood is considered normal (Pienaar 1962).

The variation in immature erythrocyte colouration is referred to as polychromasia and arises from the differing stages in haemoglobin synthesis, with hyperchromasia observed in the intense basophilic staining of immature cells, and hypochromasia observed in the pale acidophilic staining of mature/senile erythrocytes (Pendl 2006). The senescent erythrocytes in the later stages of degeneration present a different staining reaction and the loss of cytoplasm result in free erythrocytic nuclei (Pienaar 1962), or haematogones (Frye 1991). Old or senile erythrocytes are characterised by pallid staining of the cytoplasm (Pendl 2006), and increased condensing of the chromatin occurs (Mader 2000). As erythrocytes age, and increase their haemoglobin concentrations, the staining reaction of cytoplasm changes from darker basophilic in young immature cells to barely basophilic in mature cells, to a near complete loss of staining potential in senescent cells. This change is associated with the decrease in RNA and DNA, as well as increasing haemoglobin concentrations in maturing cells, as well as the decreased metabolic activity of aging erythrocytes (Hajkova *et al.* 2000).

Other red blood cell features include varied erythrocyte sizes (anisocytosis or the presence of large and small erythrocytes – macrocytes and microcytes respectively), a change in the shape of the cell (poikilocytosis) and/or nucleus, and together with polychromatic changes, provide evidence for erythropoiesis (Campbell 2004; Pendl 2006). Morphological features not associated with erythropoiesis include cytoplasmic vacuolation, the presence of intracytoplasmic inclusion bodies (visible as basophilic

spots or stippling), the absence of nuclei (erythroplastids) and spindle-shaped erythrocytes. Mitotic nuclei or binucleation have been reported in individuals showing regenerative anaemia, inflammatory response or following hibernation (Campbell 2004). Similarly, while mild levels of anisocytosis, poikilocytosis and polychromasia may be common in healthy reptiles, severe levels indicate disease (Mader 2000). Morphological changes that influence cell and nuclear structure and/or colouration can be distinguished as regenerative or degenerative changes (Pendl 2006). A regenerative response is characterised by an increase in the number of immature cells in peripheral blood and indicates erythropoietic activity. Degenerative changes incorporate those changes that are not associated with the normal developmental series of erythropoiesis, and as such, an increased degenerative response signifies a clinical abnormality.

The aims of this study were to: (1) identify and describe morphological characteristics of erythrocyte types in peripheral blood of *P. geometricus*; (2) assess differences in the erythrocyte profiles of males, females and juveniles; and (3) evaluate seasonal changes in erythrocyte profiles of cohorts.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of stained blood smears

Blood samples were obtained over four seasons at Elandsberg Nature Reserve (3 800 ha, 33° 26’ S; 19° 01’ E) in the southwestern Cape, South Africa, from 26 to 42 healthy, free-ranging geometric tortoises (including males, females and juveniles) per season (see Table 2.1 for sample sizes in different seasons). I sampled blood from unanaesthetised tortoises immediately after capture to limit stress-induced changes to blood parameters. The mass of the animals determined the maximum blood volume sampled and I took care not to exceed 0.5% of the animals’ field body mass (a conservative veterinary standard). I used a 25 G needle with a 1 or 2 ml syringe to collect blood from either the jugular vein or carotid artery. Since EDTA is known to cause lysis of chelonian cells (Harding *et al*. 2005; Knotek 2006), heparin was used as an anticoagulant, although it has been observed to impart a blue tinge to blood smears as well as affect the clumping nature of cells (Houwen 2000; Strik *et al*. 2007)**.** Sampling normally took 1 to 2 minutes and I aborted attempts if an adequate sample has not been obtained in approximately 5 minutes. The animals were kept under observation for 24 hours and during the dry season, I provided access to drinking water before returning the animals to the capture site.

I produced blood smears, in duplicate, using the wedge-smear technique with a single-use, bevel-edged glass slide spreader (Pendl 2006). Smears were air-dried, fixed in absolute methanol, and stored in dust-free boxes until being stained. I obtained best staining results from the May-Grünwald – Giemsa stains, using the technique described in Houwen (2000). To prepare the stock solutions, May-Grünwald reagent powder (0.3 g) was mixed in 100 ml absolute methanol, left to stand overnight, and subsequently filtered. Giemsa reagent powder (1 g) was mixed in 66 ml glycerol, heated to 56 ºC for 100 minutes, mixed with 66 ml absolute methanol, left to stand overnight, filtered and stored in an airtight container. I used a buffer of pH 6.8 to dilute stock solutions each time before staining; May-Grünwald stock was mixed with equal parts of buffer whereas Giemsa stock was diluted with nine parts buffer. The May-Grünwald stain was introduced to the blood smear, letting stand for 5 minutes, after which the excess solution was drained from the slide that was then introduced to the Giemsa stain for 12 minutes. The slide was then rinsed once with the buffer solution, washed in, and left to stand in distilled water for 3 minutes. Stained blood smears were left to dry and later fitted with a glass cover slip using Entellan New rapid-mounting medium for microscopy (Merck).

3.2.2 Histological evaluation and measurements

I used a Leica DM 500 photomicroscope (Leica LAS Software, Leica Microsystems Ltd., Switzerland, version 1.8.0), with 10x eyepieces, for the histological evaluation of erythrocytes under immersion oil with a 100x objective to give 1000x magnification. I assessed the size, shape and staining characteristics of cells and their nuclei for identification and a detailed description of each cell type.

In order to quantify the abundance of erythrocyte types and the occurrence of particular features, I combined erythrocyte assessments with differential white cell counts of individuals using the meandering technique. After counting 100 white blood cells, I rated the abundance of particular cell types (rubricytes, polychromatophils, senescent erythrocytes, macrocytes and microcytes), the degree of poikilocytosis, and the presence of cytoplasmic inclusions, vacuoles and parasites in the blood smear. Each item was ranked from zero to three, with zero indicating that the cell or feature was absent, and one to three representing three increasing levels of abundance: low, intermediate and high.

I used a Leica ICC50 camera linked to the Leica DM 500 digital photomicroscope (40x objective and total magnification of 400x) to take digital images of blood cells for morphometric evaluation. The images were saved as jpeg files (2048 x 1536 pixels) and then analysed using the NIKON NIS Elements (Basic Research version 3.10 Inc., Nikon Instruments, Europe B.V) imaging software. To eliminate background staining effects, the lighting contrast was increased (both high and low ranges were set to their lowest) and 'auto detect' thresholding function (from the binary toolbar) was used to select the immediate area of hue of selected pixels, thus incorporating the entire erythrocyte area. The 'erode' or 'open' function of NIS was used to precisely match cellular and nuclear boundaries with thresholded boundaries. Cellular measurements were digitally automated to a precision of 0.01 µm. Pixel size was manually calibrated using a micrometer scale automatically generated when capturing the images (at 400x and 1000x magnification, 1 pixel = 0.16 µm and 0.07 µm, respectively). Morphometric measurements were exported to Windows Excel (MS Office) and collated into one spreadsheet for statistical analysis.

In order to evaluate the effects of season and cohort on erythrocyte characteristics, I took measurements from 100 erythrocytes per individual by capturing 10 images within the mono-layered section of each bloodsmear, with each image containing at least 10 distinguishable erythrocytes. In addition, I used all the smears to identify, photograph and measure 10 representative cells of each immature erythrocyte type and of senescent cells to quantify their morphological features. Immature erythrocytes in peripheral blood included rubricytes and polychromatophils, but because the morphology of rubricytes changed substantially during development, I measured three stages of development (stages I, II and III) to represent the continuum. For each erythrocyte, I measured the following parameters of both cell and nucleus: area (surface area of the image in μm^2), perimeter (the total boundary in µm), length (the longest axis in µm), width (a derived measure calculated from area / length in µm), circularity (a measure derived from area and perimeter measurements; a circular shape has a value of 1.0 with other shapes having values <1.0), elongation (determined from Feret's diameters as MaxFeret / MinFeret) and pixelation (the statistical mean of intensity values of pixels).

3.2.3 Data and statistical analysis

SigmaStat (SPSS Inc., Chicago, U.S.A. version 2.03) was used to evaluate the data statistically. Despite various transformations, most data could not satisfy the

requirements for parametric tests and are summarised as median, 25th and 75th percentiles.

Measurements of erythrocytes were linked to specific individuals and could be evaluated for effects of season and cohort. In most instances, I could not use two-way ANOVAs because the data were not parametric, and instead used one-way ANOVAs to identify differences among seasons within each cohort and differences among cohorts within each season. When data were parametric, one-way ANOVA was followed by Student-Newman-Keuls post hoc comparisons, whereas for non-parametric data, Kruskal-Wallis ANOVA on ranks was followed by Dunn's post hoc comparisons. Because cell measurements of the immature erythrocyte stages and senescent cells were not linked to specific individuals, I used one-way ANOVAs to test for size and shape differences among these types, and separately evaluated size and shape differences among senescent cells, mature cells, and the most advanced immature cell, the polychromatophil. I used one-way ANOVAs to assess the effects of season and cohort on the prevalence (ranked from 0 to 3) of immature and aberrant erythrocytes, and specific morphological features.

Because erythrocyte cell areas varied widely, I divided the data for 100 cell areas of each individual into six size categories (≤130, 130.1-140, 140.1-150, 150.1-160, 160.1-170 and >170 μm^2) to allow more refined analyses for differences among cohorts and seasons. Because small or large cells may reflect specific physiological states, I also divided the data into fewer size classes to represent the smallest class against the remainder (≤130 versus >130 μm^2), and the largest class against the remainder (>170 versus ≤170 μm^2). Subsequently, I used Chi-square tests to evaluate if frequencies for all size classes, and for reduced size classes, differed among cohort and among season.

In all instances when multiple tests were performed, I applied sequential Bonferroni corrections to each family of tests to prevent Type I errors.

3.3 RESULTS

3.3.1 Erythrocyte types and features

Mature erythrocytes were the most common component in peripheral blood of *P. geometricus* (Fig. 3.1a). The cells were oval to elliptical with a centrally placed round-oval, dark blue-purple staining nucleus, sometimes with an irregular border. The

chromatin was visible as a coarse, evenly dispersed, strongly basophilic network. The abundant cytoplasm stained pale blue, had a homogenous texture and sometimes had basophilic inclusions (Fig. 3.1b). Occasionally, the flattened shape of erythrocytes became apparent when the edges of the cell were folded over when the smear was made (Fig. 3.1c).

Rubricytes varied greatly in size, shape and staining quality, and could be described in three distinguishable developmental stages, based mainly on staining quality and nuclear to cellular size ratios; defined as early - , intermediate - and late rubricytes (I, II and III respectively; Fig. 3.2 a – c). Transitional stages appear between all consecutive classes of development, showing a sequential development through the classes.

Rubricyte I cells were typically small (cell areas ranging from 31.43 to 120.12 μm^2) and easily mistaken for thrombocytes and/or lymphocytes. The large, irregularly shaped nucleus (ranging from 19.23 to 60.06 μm^2) stained dark blue-violet with intensely basophilic chromatin appearing coarsely stippled within darkly basophilic parachromatin (Fig. 3.2a). The sparse, hyperchromatic cytoplasm stained slightly less intense blue than the nucleus. Nuclear to cellular size ratios were characteristically high in this stage (43.6% – 63.1% in range).

The intermediate rubricyte II cells (cellular areas from 50.51 to 144.26 μm^2) displayed an increasing amount of cytoplasm, together with an irregular decrease in basophilic intensity (Fig. 3.2b). The increase in cytoplasm resulted in a decreasing nuclear to cellular size ratio (ranging from 37.0% – 48.6%). In this stage, nuclei ranged from 19.43 to 70.15 μm^2 in area. The still darkly basophilic chromatin appeared to contract, appearing increasingly clumped within less intensely basophilic parachromatin.

In the advanced rubricyte III stage of rubricyte development, the cells tended to be large (cell area ranged of 89.87 to 169.0 μm^2), with still increasing amounts of cytoplasm which sometimes appeared ‘folded’ and stained less intensely basophilic (Fig. 3.2c). The nuclear to cell area ratio continued to decrease, ranging from 24.3% to 37.5% in this stage. The basophilic nucleus ranged from 27.91 to 53.97 μm^2 in size. The chromatin appeared condensed and both chromatin and parachromatin stained less intensely.

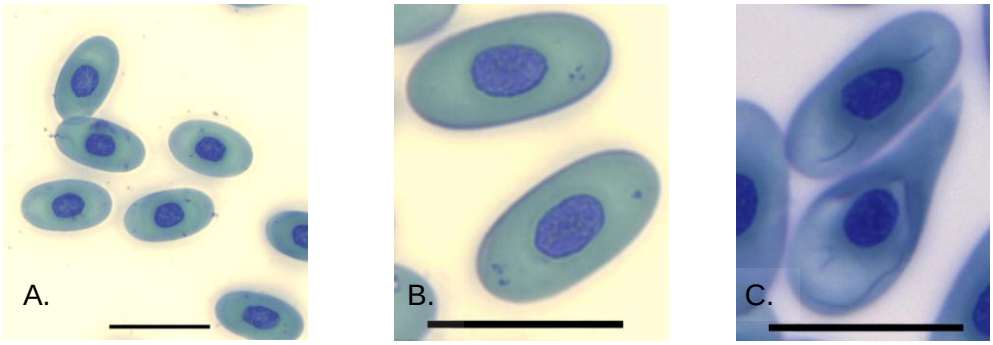

Figure 3.1 Mature erythrocytes of *Psammobates geometricus* with condensed chromatin in the nucleus (a, b). Note basophilic inclusion bodies in cytoplasm (b). Edges of the flattened cells can fold over when making smears (c). Scale represents 20 µm, 1000x magnification, Romanowsky stains.

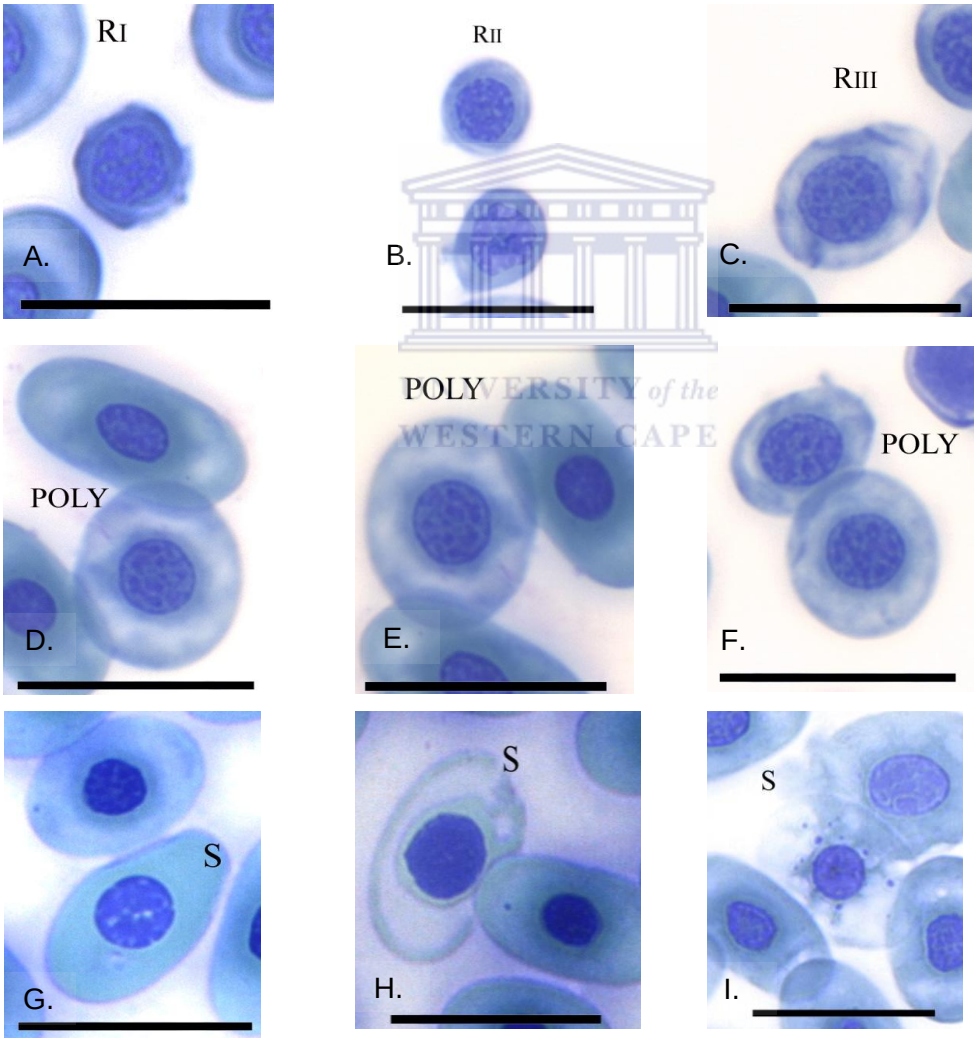

Figure 3.2 Immature and senescent red blood cells of *Psammobates geometricus* showing three rubricyte developmental stages (a - c), polychromatophilic erythrocytes (d - f) and senile erythrocytes (g - i). Scale represents 20 µm, 1000x magnification, Romanowsky stains.

Polychromatophils typically appeared larger (116.87 to 243.53 μm^2) than early rubricytes, and rounder than mature erythrocytes. The faintly basophilic cytoplasm did not stain uniformly, but instead had a mottled appearance, indicating the patchy accumulation of haemoglobin and the reduction of nucleic acids in the cytoplasm (Fig. 3.2 d-f). Polychromatophil nuclei measured 22.34 to 45.68 μm^2, were roundish, and the chromatin still appeared clumped with an increase in lighter-staining parachromatin. Nuclear to cellular ratios were low (17.8% to 27.9%) compared to other immature cells. As the cells matured from this stage, elongation increased so that the cell became oval to ellipsoid, cytoplasmic staining became more homogenous, the nuclei tended to contract and elongate, and the chromatin became denser.

Senile or senescent erythrocytes were large with cell areas ranging from 187.56 to 301.03 μm^2 and nuclei between 39.92 and 77.08 μm^2. Nuclear to cellular ratios were between 16.3% and 34.6%. Two types of cellular degeneration could be distinguished. The most common form of senescence was in which the cell and nucleus “swell” as they degenerated, with both displaying an increasing loss in staining potential, giving the appearance of ‘ghost cells’. When cytoplasm was still visible, it stained a pale light blue, almost clear, while the nucleus stained pale lilac (Fig. 3.2 g,h). Often, the cytoplasm disintegrated, releasing the nucleus, which appeared as lilac stains in the smear. Less typically observed in the degeneration of erythrocytes was the increased condensing and pyknosis of the nucleus, with the cytoplasm “imploding” around it until it disappeared, releasing the very dark, round nucleus (Fig. 3.2i).

Present but in consistently low occurrence in peripheral blood were macrocytes (Fig. 3.3a), whereas microcytes were not observed. Erythrocyte inclusion bodies were noted as darkly basophilic-staining small cytoplasmic spheres and/or rods (Fig. 3.3b), often associated with cytoplasmic vacuolation (Fig. 3.3b). Also observed were mitotic erythrocytes in various stages of mitosis (Fig. 3.3c). Poikilocytosis (Fig. 3.3d) was not common and no evidence of blood parasites was observed; this includes extra-cellular and intra-erythrocytic haemoparasites.

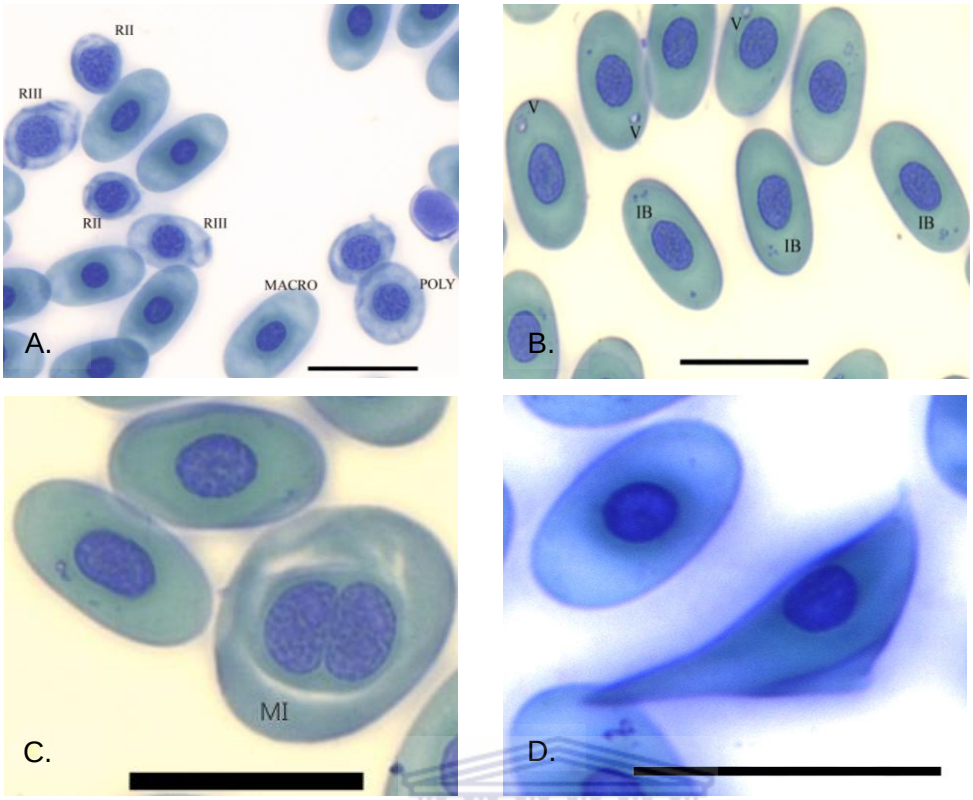

Figure 3.3 Red blood cells of *Psammobates geometricus* showing erythrocyte diversity in circulation (a). Note macrocyte (MACRO), rubricytes (RII, RIII), polychromatophil (POLY), cytoplasmic inclusion bodies (IB), vacuoles (V; b), mitotic erythrocyte (MI; c) and poikilocytosis (d). Scale represents 20 µm, 1000x magnification, Romanowsky stains

Statistical comparisons of size and shape of immature and senescent erythrocytes (Table 3.1) indicated that stage III rubricytes and polychromatophils had larger areas and were longer and wider than stages I and II rubricytes ($F_{4,45} > 14.13$, $P < 0.0001$). Senescent cells had larger areas and lengths than all immature stages, but their widths did not differ from those of polychromatophils. Nuclear to cellular area ratios decreased progressively as cells matured, but did not differ between polychromatophils and senescent erythrocytes ($F_{4,45} = 73.30$, $P < 0.0001$). Senescent erythrocytes were less circular, and more elongated, than immature cells, with no differences among the immature stages ($F_{4,45} > 17.70$, $P < 0.0001$). When comparing cell areas of the complete data set, representing mostly mature erythrocytes, with those of the most advanced immature (polychromatophil) and senescent cells, I found that mature cells had smaller cell areas than senescent cells but did not differ from polychromatophils ($H_2 = 28.18$, $P < 0.0001$). Mature and

senescent cells were more elongated, or less circular, than polychromatophils (H_2 > 18.19, P < 0.0005). The nuclear to cellular ratio was lower in mature cells than in senescent cells and polychromatophils (H_2 = 46.63, P < 0.0001).

Erythrocyte nuclei of senescent cells had larger surface areas (Table 3.1), lengths and widths than immature cells had, but nuclear measurements did not differ among immature cell stages ($F_{4,45}$ > 4.80, P < 0.002). Mature erythrocyte nuclei had smaller areas, lengths and widths than both polychromatophils and senescent erythrocytes (H_2 > 35.38, P < 0.0001). Nuclear circularity and elongation did not differ among immature and senescent cells (P > 0.11), whereas nuclei of mature erythrocytes were more elongated (H_2 = 38.55, P < 0.0001) and tended to be less circular (H_2 = 6.11, P = 0.047, post hoc comparisons not significant) than those of polychromatophils or senescent erythrocytes.

Table 3.1 Erythrocyte cellular and nuclear sizes, and circularities, as well as the ratio of nuclear to cellular (N/C) areas in geometric tortoises. Data are mean ± standard deviation for immature developmental stages and senescent erythrocytes, measured from ten cells of each type.

	Cell area (μm^2)	Nuclear area (μm^2)	N/C area ratios (%)	Cell circularity	Nuclear circularity
Rubricyte I	70.73 ± 28.11	36.26 ± 13.35	52.33 ± 6.56	0.95 ± 0.02	0.92 ± 0.04
Rubricyte II	96.88 ± 34.34	40.73 ± 17.58	41.14 ± 3.87	0.94 ± 0.02	0.94 ± 0.03
Rubricyte III	133.80 ± 25.79	41.88 ± 7.85	31.64 ± 4.33	0.93 ± 0.04	0.96 ± 0.03
Polychromatophil	161.28 ± 47.24	32.22 ± 8.02	20.37 ± 3.32	0.94 ± 0.03	0.95 ± 0.02
Senescent Cell	232.32 ± 36.44	56.17 ± 13.60	24.29 ± .5.19	0.86 ± 0.03	0.95 ± 0.02

3.3.2 Prevalence of erythrocyte types and features

In addition to the mature erythrocytes, younger as well as senile erythrocytes were present in circulation throughout all seasons and cohorts (Fig. 3.3a); however, the seasonal composition of erythrocyte developmental stages differed. For female tortoises, rubricytes were more abundant in winter and spring than in autumn (H_3 = 15.61, P = 0.0014) whereas polychromatophils were more abundant in winter than in summer, and both winter and spring values were higher than autumn values (H_3 = 26.50; P < 0.0001). While males showed no significant seasonal effect on rubricytes (P = 0.241), polychromatophils were more frequent in winter than in autumn (H_3 = 13.05, P = 0.0045). Season did not influence the abundance of immature cells in

juveniles (P > 0.149), and within seasons, the abundance of rubricytes or polychromatophils did not differ among cohorts (P > 0.138). A two-way ANOVA was possible when rubricyte and polychromatophil counts were combined. The abundance of immature erythrocytes changed with season ($F_{3,114}$ = 12.80, P < 0.0001), with winter and spring values exceeding summer and autumn values, while cohort had no effect on immature erythrocyte abundance and cohort and season did not interact (P > 0.15).

Data for senescent erythrocytes failed normality but two-way ANOVA is particularly robust for normality violations (Zar 1999). The test showed that although cohort had no effect on senescent erythrocyte abundance (P = 0.890), season had a strong effect ($F_{3,114}$ = 14.29, P < 0.0001), with a higher occurrence of senescent cells in autumn than in the other seasons. These results were confirmed by multiple one-way ANOVAs, in which this trend applied to adult tortoises only, although the female P-value did not pass the corrected Bonferroni value.

Degrees of poikilocytosis did not differ among season in adult geometric tortoises (P > 0.161), but was significant for juveniles (H_3 = 12.30, P = 0.0064), which showed a tendency towards increased poikilocytosis in autumn despite not having significant post hoc differences. Within-season analyses revealed that juveniles tended to have a higher degree of poikilocytosis than males or females had in autumn (H_3 = 8.07, P = 0.018 was higher than adjusted Bonferroni P of 0.0083); there were no cohort differences within other seasons (P > 0.097).

There were no significant effects of cohort within each season, or season within each cohort, upon the abundances of macrocytes, vacuoles and inclusion bodies (P > 0.059), although males and juveniles tended to have more erythrocytic inclusion bodies in autumn than in other seasons (H_3 = 9.62 and 9.32, P = 0.022 and 0.025, respectively; required adjusted Bonferroni P = 0.0071).

3.3.3 Erythrocyte size and shape

Erythrocyte cell size (surface area) ranged from 52.88 to 274.46 μm^2 (Fig. 3.4a). Male erythrocytes were larger than those of juveniles and females in summer and autumn (H_2 > 17.75, P < 0.0005), while in winter, male and juvenile erythrocytes were larger than those of females (H_2 = 53.42, P < 0.0001). Erythrocytes in spring were largest in juveniles, followed by males, and smallest in females (H_2 = 85.94, P < 0.0001). Within males, erythrocytes were equally large in winter and spring, followed

by autumn, with smallest erythrocytes in summer (H_3 = 77.61, P < 0.0001). Erythrocytes in both females and juveniles were largest in spring, followed by winter, and equally small in autumn and summer (H_3 > 140.36, P < 0.0001).

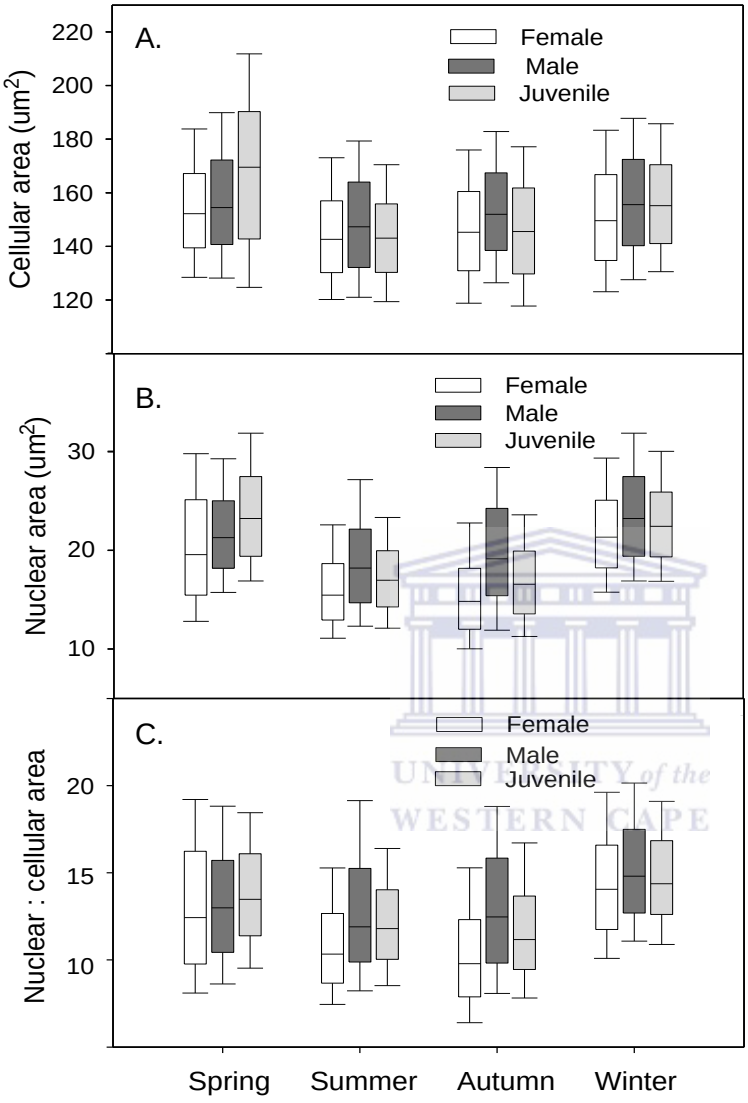

Figure 3.4 Seasonal changes in male, female and juvenile geometric tortoise erythrocyte cellular and nucleus surface areas (μm^2), as well as nuclear to cellular area ratios, measured from 100 cells per animal. Box plots contain the median, 25th and 75th quartiles, while error bars represent the 5th and 95th percentiles. Seasonal sample sizes are indicated in Table 2.1.

Erythrocyte nuclei ranged from 4.10 to 49.51 μm^2 (Fig. 3.4b). Nuclear areas in summer, autumn and winter showed the same pattern: male nuclei were largest, followed by juveniles, and female nuclei were smallest (H_2 > 80.11, P < 0.0001). In

spring however, juveniles had larger nuclei than adult tortoises had (H_2 = 61.72, P < 0.0001). Nuclear areas were larger in winter and spring than in summer and autumn for all cohorts (H_3 > 398.36, P < 0.0001); the patterns were Wi>Sp>Au>Su for males, Wi>Sp>Su=Au for females, and Wi=Sp>Su=Au for juveniles.

Nuclear to cellular area ratios ranged between 3.05% and 31.59% (Fig. 3.4c) and differed among cohorts within seasons (H_2 > 10.06, P < 0.0065), and among seasons within cohorts (H_3 > 313.5, P < 0.0001). Spring ratios were highest in juveniles, with no differences between adults. Summer ratios of juveniles and males were higher than in females, winter ratios for males were higher than in juveniles and females, whereas autumn ratios were highest in males, followed by juveniles, and females had lowest nuclear to cellular ratios. All cohorts followed a similar pattern of nuclear to cellular ratios with high values in winter and spring. In males, winter ratios were higher than spring, summer and autumn, with spring values being higher than values in autumn. Female ratios were highest in winter, followed by spring, then summer, and lowest in autumn, while juveniles showed equally lowest ratios in summer and autumn, preceded by spring, and largest ratios in winter.

Erythrocyte lengths ranged between 9.92 and 33.44 µm while widths ranged from 4.12 to 14.17 µm (Table 3.2); both lengths and widths showed seasonal (H_3 > 18.01, P < 0.0004) and cohort (H_2 > 55.96, P < 0.0001) differences, except for widths in summer and autumn (P > 0.081). Erythrocytes in winter, summer and autumn were longer in males than in juveniles and females with female erythrocytes being longer than those of juveniles in autumn. In the spring, juveniles had the longest erythrocytes, followed by males and then females. Erythrocyte widths showed significant differences only in winter and spring, in which they followed the same pattern, with juveniles showing widest erythrocytes, and no difference between adult erythrocyte widths. Within males, erythrocytes were longer in autumn and winter than in summer and spring. Females' erythrocytes were longer in autumn than in spring and summer, while winter lengths were higher than in summer. In juveniles, erythrocytes were longer in spring than in other seasons, and erythrocytes in winter were longer than in summer. Erythrocytes were widest in spring, followed by winter; in females and juveniles, autumn and summer widths did not differ but males had wider erythrocytes in autumn than in summer.

Table 3.2 Dimensions (medians, 25th and 75th quartiles) of erythrocyte cells (C) and nuclei (N) in female, male and juvenile geometric tortoises over spring, summer, autumn and winter, measured from 100 cells per animal.

	Females			Males			Juveniles		
	Med	25%	75%	Med	25%	75%	Med	25%	75%
C-Length									
Spring	17.8	16.8	18.9	18.0	17.0	19.2	18.6	17.2	20.0
Summer	17.7	16.6	18.8	18.2	17.2	19.2	17.6	16.6	18.7
Autumn	18.1	17.0	19.1	18.7	17.5	19.7	17.8	16.6	18.9
Winter	17.9	16.8	19.1	18.5	17.4	19.6	17.8	16.9	18.9
C-Width									
Spring	8.5	8.0	9.3	8.6	8.0	9.4	9.2	8.3	9.8
Summer	8.1	7.6	8.7	8.1	7.6	8.7	8.1	7.6	8.6
Autumn	8.1	7.5	8.8	8.2	7.7	8.8	8.2	7.6	8.8
Winter	8.3	7.8	8.9	8.4	7.8	9.1	8.7	8.1	9.4
C-Elongation									
Spring	1.7	1.5	1.8	1.7	1.5	1.9	1.7	1.5	1.8
Summer	1.8	1.6	2.0	1.8	1.7	2.0	1.8	1.6	1.9
Autumn	1.8	1.7	2.0	1.9	1.7	2.0	1.8	1.6	1.9
Winter	1.7	1.6	1.8	1.8	1.6	1.9	1.6	1.4	1.8
C-Pixelation									
Spring	190	180	201	192	1808	200	192	185	199
Summer	192	184	198	178	169	189	191	180	201
Autumn	194	182	201	188	171	202	184	173	198
Winter	181	171	190	180	168	190	180	172	186
N-Length									
Spring	6.0	5.3	6.7	6.2	5.6	6.8	6.6	5.9	7.4
Summer	5.5	5.0	6.1	6.0	5.4	6.7	5.8	5.3	6.3
Autumn	5.4	4.8	6.1	6.1	5.5	6.8	5.6	5.1	6.2
Winter	6.3	5.8	6.8	6.5	6.0	7.1	6.5	6.0	7.1
N-Width									
Spring	3.3	2.9	3.8	3.3	2.9	3.7	3.4	3.0	3.7
Summer	2.8	2.5	3.2	3.0	2.7	3.4	2.9	2.6	3.3
Autumn	2.8	2.4	3.1	3.1	2.8	3.6	3.0	2.6	3.3
Winter	3.4	3.1	3.8	3.5	3.2	3.9	3.5	3.2	3.8
N-Elongation									
Spring	1.3	1.2	1.5	1.4	1.3	1.5	1.5	1.4	1.6
Summer	1.5	1.3	1.6	1.5	1.4	1.7	1.5	1.4	1.7
Autumn	1.5	1.3	1.6	1.5	1.3	1.6	1.4	1.3	1.6
Winter	1.4	1.3	1.5	1.4	1.3	1.5	1.4	1.3	1.6
N-Pixelation									
Spring	153	141	164	148	134	161	153	146	162
Summer	149	137	159	134	125	146	153	140	164
Autumn	150	136	161	141	129	153	142	133	152
Winter	120	111	128	123	115	133	122	116	130

Erythrocyte nuclei lengths ranged between 2.72 and 12.58 µm and widths ranged between 1.42 and 5.91 µm (Table 3.2). Nuclear lengths and widths were influenced by season ($H_3 > 200.7$, $P < 0.0001$) and by cohort ($H_2 > 7.59$, $P < 0.022$). Nuclei were longer in males and juveniles than in females in winter, whereas in spring, they were longest in juveniles, followed by males and then females. In the summer and autumn, erythrocyte nuclei were longest in males, followed by juveniles and shortest in females. Nuclei were widest in males in winter, summer and autumn, as well as being narrowest in females in summer and autumn; in winter, there was no difference between juvenile and female nuclei widths. In spring, juveniles had the widest nuclei, but widths did not differ between males and females. Within males, winter nuclei were longer than in other seasons whereas female nuclei were longest in winter, followed by spring, and equally short in summer and autumn. Juvenile erythrocyte nuclei were longest in spring and winter, followed by summer and shortest in autumn. Among all cohorts, nuclei were widest in winter, followed by spring; in males, nuclei were wider in autumn than in summer, but summer and autumn widths did not differ in females and juveniles.

Erythrocyte circularity ranged from 0.332 to 1.000 (Fig. 3.5a) and elongation from 1.06 to 2.87 (Table 3.2). Both circularity and elongation showed effects of season ($H_3 > 172.2$, $P < 0.0001$), and cohort ($H_2 > 19.51$, $P < 0.0001$), except for circularity in spring ($P = 0.129$). Erythrocytes in juveniles were more circular than those of males in summer, autumn and winter, with male erythrocytes in autumn being more circular than in females, and female erythrocytes being more circular than those of males in winter. The reciprocal pattern was valid for elongation in winter, but male and female elongation did not differ in autumn, and males had more elongated erythrocytes than females had in spring. In summer, elongation was greatest in males and smallest in juveniles. Within males, erythrocytes were roundest in spring, followed by winter, and were least round in summer and autumn. Elongation showed the same trend but erythrocytes were more elongated in autumn than in summer. Female erythrocyte circularities were higher in winter and spring than in summer, followed by autumn. Again, elongation reflected the same pattern but winter erythrocytes were more elongated than spring erythrocytes. Juvenile erythrocytes were roundest in winter, followed by spring and were least round in summer and autumn, with a reciprocal pattern for elongation.

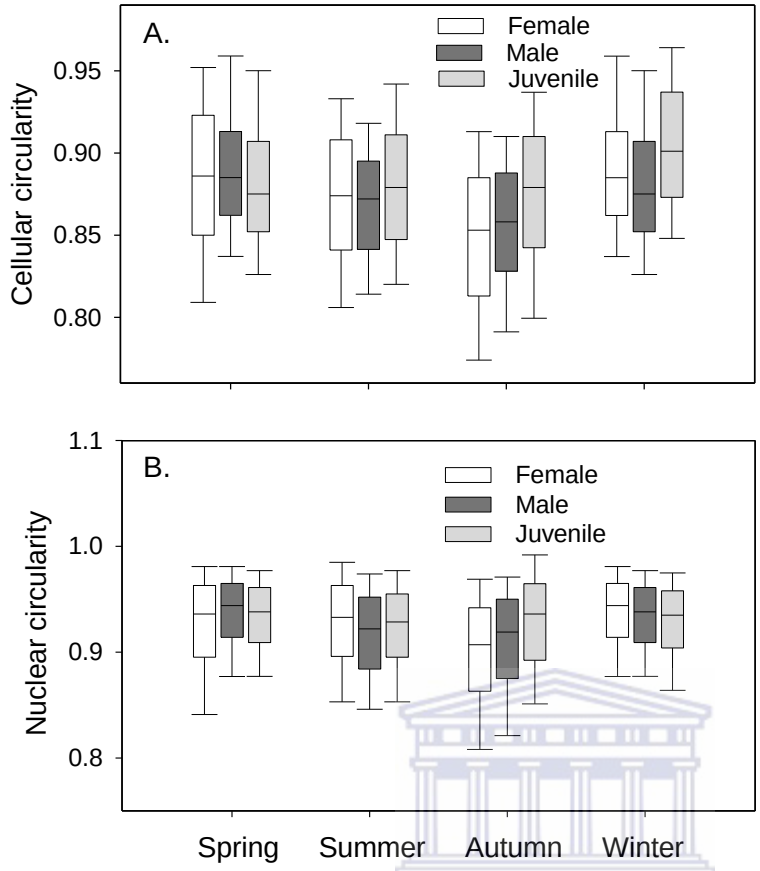

Figure 3.5 Seasonal changes in circularity (where 1.0 represents a sphere) of erythrocyte cells (a) and nuclei (b) of *Psammobates geometricus*, measured from 100 cells per animal. Box plots contain the median, 25th and 75th quartiles, while error bars represent the 5th and 95th percentiles. Seasonal sample sizes are indicated in Table 2.1.

Erythrocyte nuclei circularity ranged from 0.35 to 1.00 (Fig. 3.5b) and elongation from 1.03 to 3.30 (Table 3.2). Both circularity and elongation were affected by season (H_3 > 55.87, P < 0001) and cohort (H_2 > 14.15, P < 0008). In spring, nuclei in adults were rounder than those in juveniles, but the pattern did not correspond for elongation; males had more elongated nuclei than females had. In winter, female nuclei were rounder than those in males, with both being rounder than juvenile nuclei. The reciprocal, elongation, yet again did not mirror results for circularity; although juvenile nuclei were more elongated than those of adults, elongation did not differ for males and females. In autumn, juvenile nuclei were roundest, followed by males, and female nuclei were least round. Male and female elongation did not differ, but their nuclei were more elongated than those of juveniles were. Summer nuclear

circularities were larger in females and juveniles than in males, whereas male and juvenile elongation did not differ but were more pronounced than in females. Within males, winter and spring nuclei circularities were larger than summer and autumn circularities, but elongation decreased progressively from summer to autumn, spring and winter. In females, circularity was higher in winter than in spring, summer and autumn, with spring and summer being higher than autumn. Elongation was more pronounced in autumn and summer than in spring and winter. Erythrocyte nuclei in juveniles were more circular in autumn, winter and summer than in spring, whereas the nuclei were more elongated in summer and spring than in autumn and winter.

Erythrocyte and nuclear pixelation (Table 3.2) was influenced by cohort ($H_2 > 6.99$, $P < 0.030$) and season ($H_3 > 382.4$, $P < 0.0001$). In summer, females and juveniles had higher pixelation values than males had, while in autumn, female values were higher than those of males, and juveniles had lowest pixelation values. In winter, females had higher values than males had, whereas juvenile values were higher than those of males in spring. Within males, pixelation values were highest in spring, followed by autumn, while winter and summer values were equally low. In females, pixelation was lowest in winter whereas in juveniles, erythrocyte pixelation was highest in spring, followed by summer, then autumn, with lowest pixelation in winter.

Nuclear pixelation followed similar patterns in spring and summer: juvenile values were higher than those in females, and males showed lowest values. In autumn, female nuclear pixelation was higher than that in males and juveniles. In winter however, males and juveniles had higher nuclear pixelation intensity than in females. Within males, pixelation values in nuclei were largest in spring, followed by autumn, then summer and lowest in winter. Female nuclear pixelation was higher in spring than in autumn, summer and winter, and autumn and summer values were higher than winter values. In juveniles, nuclear pixelation was highest in spring and summer, followed by autumn, and lowest in winter.

3.3.4 Proportional representation of erythrocyte size classes

The frequency distribution of the six erythrocyte size classes differed among cohorts within each season ($\chi^2_5 > 15.3$, $P < 0.009$), except that females and juveniles did not differ in summer and autumn ($P > 0.52$). The size class frequencies differed among seasons for males, females, and juveniles ($\chi^2_5 > 13.8$, $P < 0.016$) except between autumn and summer for females and between winter and spring for males ($P > 0.17$).

The seasonal pattern of abundance for small erythrocytes (< 130 μm^2) relative to the other size classes was relatively similar for females and juveniles: for both cohorts, small erythrocytes were most abundant in summer and autumn, which did not differ (P > 0.611). Juveniles had fewer small cells in spring and the least in winter (χ^2_1 > 10.36, P < 0.0001; Fig. 3.6). In females, small erythrocytes were more abundant in summer than in spring and winter, whereas autumn counts were higher than in spring, but did not differ from those in winter (χ^2_1 > 10.02, P < 0.002). Males had the largest frequency of small cells in summer (χ^2_1 > 20.26, P < 0.0001) with no difference among the other seasons (P > 0.19). There were no cohort differences in the relative abundance of small erythrocytes during spring and summer (P > 0.103). However, in autumn, females and juveniles had a higher relative abundance of small cells than males had (χ^2_1 > 33.50, P < 0.001), whereas in winter, females had the higher relative abundance of small cells, followed by males, and juveniles had the least abundance (χ^2_1 > 7.53, P < 0.006; Fig. 3.6).

Males and females had the same seasonal pattern of abundance for the largest erythrocyte size class (> 170 μm^2) relative to the other size classes (χ^2_1 > 10.40, P < 0.002; Fig. 3.6). The relative frequency for large erythrocytes was equally high in winter and spring (P > 0.66) and lower in autumn and summer, although these two seasons did not differ after applying a Bonferroni correction (P > 0.032). Juveniles had the highest proportion of large erythrocytes in spring (χ^2_1 > 27.37, P < 0.0001), followed by winter, then autumn and summer, which did not differ following Bonferroni corrections (P = 0.042). Within seasons, large erythrocyte frequencies differed among cohorts, with a similar pattern in summer and autumn, where males' frequencies were higher than in females and juveniles (χ^2_1 > 10.57, P < 0.001). In spring, juveniles had the highest frequency of large erythrocytes, followed by males and then females (χ^2_1 > 11.79, P < 0.001). In winter, the relative abundance of large cells did not differ for juveniles and males, both of which were higher than that in females (χ^2_1 > 7.88, P < 0.005).

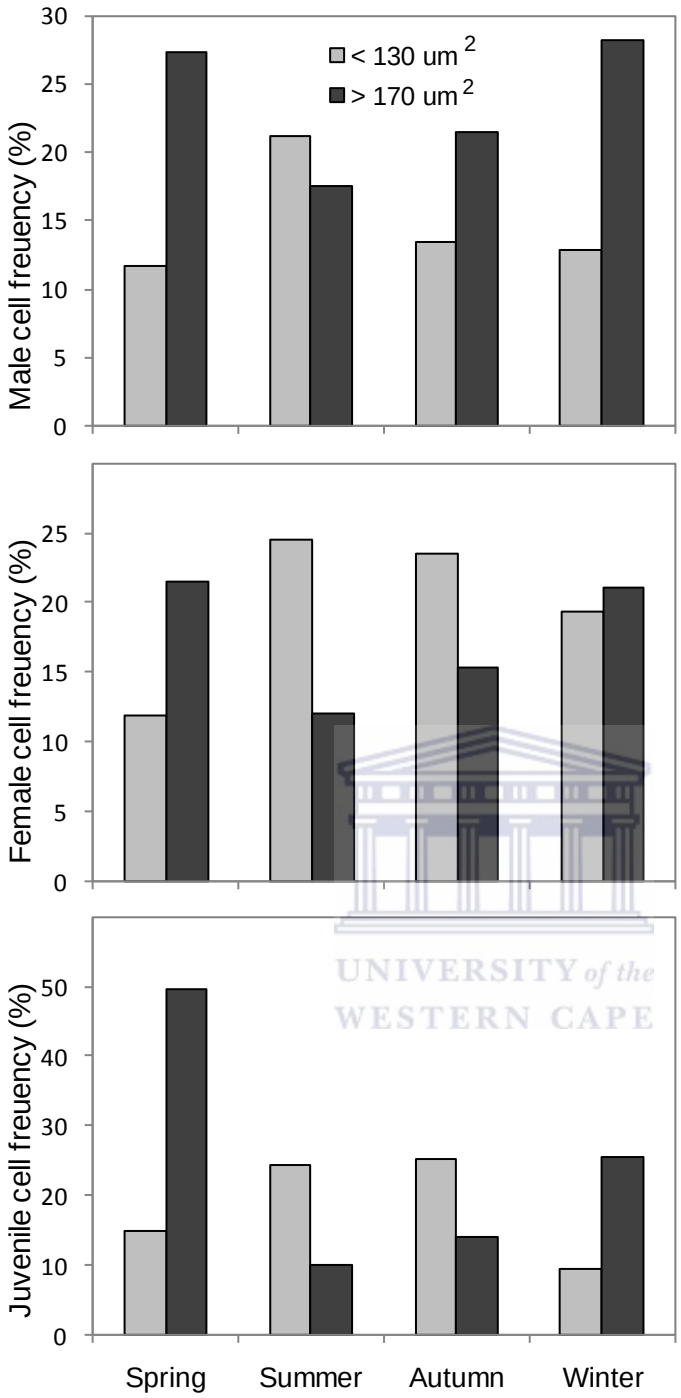

Figure 3.6 Seasonal proportional representation of small (<130 µm^2) and large (> 170 µm^2) erythrocyte cell sizes of male, female and juvenile geometric tortoises, measured from 100 cells per animal.

3.4 DISCUSSION

3.4.1 Erythrocyte development, morphology and profiles

Evaluation of blood cell morphology and characteristics can reflect the state of physiological processes (Arikan & Cicek 2010). External factors such as temperature and internal factors that influence the activity level of individuals, such as reproductive status, have great effects on the physiology of ectotherms, which is reflected in the wide range of interspecific and intraspecific variation of reptilian red blood cell morphology (Frair 1977; Campbell 2004; Strik *et al*. 2007). Exacerbating this wide variation has been the inconsistencies in identification and nomenclature of red blood cell types.

While Bernstein (1938) describes early and late erythroblasts as well as normoblasts, Pienaar (1962) continues to include basophilic as well as polychromatic normoblasts to his description of erythrocyte developmental stages, which are also used by Vasse & Beaupain (1981). These terms have been modified to rubriblasts and pro-, meta- and rubricytes by certain authors, whereas most authors describe simply immature and / or polychromatic erythrocytes (Alleman *et al*. 1992; Hajkova *et al.* 2000). My classification of immature erythrocytes into the three rubricyte stages and polychromatophils incorporates nomenclature used in modern literature (Strik *et al*. 2007) and identifies recognisable changes in early (rubricyte) immature erythrocyte morphology.

Mature erythrocytes can result from a variety of pathways (Pienaar 1962; Frye 1991; Pendl 2006), and although certain authors refer to the pluripotentiality of thrombocytes (Pendl 2006), some refer to the pluripotentiality of lymphocytes (Saint Girons 1970). Frye (1991) related the pluripotentiality of thrombocytes to the development of mature erythrocytes, whereas Pienaar (1962) describes rather lymphoid-type pro-erythrocytes, as well as stem-cell pro-erythrocytes, which are loosely described as “Type V cells” by Hajkova *et al.* (2000) and Knotkova *et al.* (2005).

Erythrocytes in circulating *P. geometricus* blood were observed to exist in immature, mature and senescent stages of development, as is common in reptiles (Pienaar 1962; Campbell 2004), indicating a co-existence of all maturation stages. Immature erythrocytes were identified occurring from the earliest stage rubricytes, to the latest developmental stage, the polychromatophils, with all stages in the continuum

represented. As cells matured from earliest rubricytes to later polychromatophils, an increase in cellular area, together with declining nuclear to cellular area ratios was observed. This suggests that rubricytes increased in size by cytoplasmic increase and that nuclear shrinking appeared evident only in the polychromatophils. As polychromatophils developed into mature erythrocytes, cell size did not change, although cells became more elongate, with further shrinking and elongation of the nucleus. Senescence in mature erythrocytes was characterised by increased cellular and nuclear swelling.

A number of authors report immature erythrocytes of reptiles and tortoises being smaller than mature erythrocytes (Frair 1977; Reavill 1994; Mader 2000), while Bernstein (1938) described immature erythroblasts of *Testudo geometrica* having larger cellular and nuclear lengths and widths than the mature erythrocytes. Zhang *et al.* (2011) describe chelonian immature erythrocytes only as being rounder, with rounder nuclei than mature erythrocytes. Immature erythrocytes in *P. geometricus* appear similar to those in the Desert tortoise (*G. agassizii;* Alleman *et al.* 1992) as being smaller than mature erythrocytes, however, with larger nuclei, and my findings suggest that nuclear to cellular area ratios are more indicative of erythrocyte maturity, compared to cellular and nuclear sizes.

While there have been no reports of cohort differences in immature erythrocyte occurrences, it is reported that juvenile reptiles have a high occurrence of immature erythrocytes in circulation (Pienaar 1962; Mader 2000; Campbell 2004). My results indicate no significant differences in seasonal abundances of immature erythrocytes in juvenile geometric tortoises, and could be attibuted to low sample size.

Erythrocyte profiles showed no significant effect of cohort within seasons, but erythrocyte maturation stages showed significant seasonal changes. The proliferation of immature erythrocytes in winter and spring indicate a regenerative (erythropoeitic) response in geometric tortoises during winter and spring. Erythrocyte morphometrics during these sampling seasons reveal that nuclear to cellular ratios were highest for all cohorts, suggesting that high nuclear to cellular ratios can indicate the presence of immature cells. This regenerative erythropoietic response is common to ectotherms emerging from a period of limited metabolic activity brought about by low environmental temperatures (Pienaar 1962; Dessauer 1970).

Senescent erythrocytes were more abundant in circulation in autumnn. Intra-erythrocytic inclusion bodies and vacuoles in geometric tortoise blood were observed without any significant seasonal or cohort differences, however with a tendency towards increased prevalence during the drier autumn months in juveniles and males. Reavill (1994) describes such basophilic bodies, sometimes being associated with cytoplasmic vacuoles as common in the Desert tortoise; they are noted in chelonian erythrocytes by Zhang *et al.* (2011) without evidence for illness, as in blood cells of the Green turtle (Casal & Oros 2007), and are believed to be remnants of organelle degeneration (Work *et al.* 1998). In addition to this, and also occurring in autumn, was the tendency towards poikilocytosis. The increased presence of erythrocyte inclusion bodies, senesence and poikilocytosis is indicative of a degenerative response (Pendl 2006) and suggests stress conditions during the dry season, which correspond to low body conditions for all cohorts in autumn (see Table 2.2).

3.4.2 Erythrocyte dimensions and shape

Since erythrocytes exist in circulation throughout the full range of developmental stages, it was impossible to exclude immature erythrocytes from the selection when making erythrocyte measurements. It is likely that smaller cells incorporated rubricytes and larger cell classes included polychromatophils and senescent cells.

Understanding size and shape characteristics of erythrocytes is important, since the surface area to volume ratio of red blood cells is a critical determining factor for gaseous exchange in the tissues. Thus, smaller cells have a larger surface to volume ratio and are more efficient in gas exchange than larger cells, and similarly, elliptical cells provide a larger surface area for gaseous exchange than round cells (Hartman & Lessler 1964; Shadkhast *et al.* 2010).

Erythrocyte measurements of *P. geometricus* obtained in this study are similar to those in *Testudo geometrica* (Bernstein 1938), in which average erythrocyte lengths and widths were reported at 18 and 10 µm, with slightly higher nuclei lengths and widths at 8 and 4 µm respectively. When compared to blood cells of *T. graeca* (Arikan & Cicek 2010), with lengths and widths of cells at 17.4 and 12.0 µm, and nuclei at 6.1 and 4.9 µm, erythrocytes and nuclei of geometric tortoises appeared to be more elongate. *Psammobates geometricus* erythrocyte ranges are within those described for *Agrionemys horsfieldii* (Hajkova *et al*. 2000) which have lengths and

widths measuring 19.5 and 9.2 µm, with nuclei measuring 6.4 µm in length and 3.6 µm in width.

3.4.3 Seasonal changes in erythrocyte morphology

Owing to the direct influence of external environmental factors on ectothermal homeostatic regulation, seasonal influences can be reflected in changes in red blood cell morphology (Campbell 2004; Strik *et al*. 2007).

Erythrocytes in *P. geometricus* showed evidence of haemodilution in winter and spring, where larger cell size classes dominated in circulation. Hydration following the rainfall events (Fig. 2.3) is likely to have caused an increase in blood plasma and erythrocyte cytoplasm, accounting for largest cellular and nuclear areas during these sampling periods. Added to this, cellular morphometrics revealed that erythrocyte circularity was hightest for all cohort during winter and spring, suggesting that high cellular circularity can represent haemodilution conditions. Similarly, during the drier seasons, haemoconcentration likely resulted in cellular shrinking, and a proliferation of the smallest erythrocyte size class is evident for males, females and juveniles.

Seasonal hydration fluctuations can be observed through variations in packed cell volume, as plasma volume is influenced by hydration state. Geometric tortoise PCV, however, showed little evidence of dehydration in the dry season (see Chapter 2.4.1). This is typical in certain arid or semi-arid tortoises, which have evolved anatomical and physiological adaptations enabling them to thrive under water-restricted conditions. This anhomeostasis of body plasma is observed and described in the Desert tortoise (*G. agassizii*, Peterson 1996; Peterson 2002). Since packed cell volumes are also affected by red cell count and size, it is difficult to identify responses to isolated factors.

During the dry summer and autumn, smaller and more elongate erythrocytes as well as nuclei dominate in circulating blood. Elongation of the nuclei is reported to provide a larger surface area exchange with haemoglobin-rich cytoplasm (Shadkhast *et al*. 2010), thus rendering them more efficient during these warmer, drier months. This increased efficiency of erythrocytes would not only serve to accommodate elevated metabolic rates, which in ectotherms, result from higher temperatures in the dry seasons (Dessauer 1970), but also enables increased physiological processes brought about by favourable conditions following winter rainfall. These include an increase in foraging opportunities as well as mating behaviour typically observed in

P. geometricus in the drier seasons (Hofmeyr & Henen, unpublished data) and have been described for *G. agassizii* (Henen *et al*. 1998; Christopher *et al*. 1999; Dickinson *et al*. 2002).

3.4.4 Cohort differences in erythrocyte morphology

Erythrocyte size differences between males and females have been reported in Caspian turtles (Metin *et al*. 2008), but not in the terrapin *Emys orbicularis* (Colagar & Jafari 2007) nor in Russian tortoises *T. horsfieldii* (Shadkhast *et al*. 2010). Worth noting, is that these results have been based on studies with comparably smaller sample sizes. Results obtained in this study indicate that erythrocytes of *P. geometricus* differ among cohort, and these differences are attributed to differences in growth and/or reproductive patterns.

In male tortoises, erythrocyte differences were more pronounced in the summer and autumn, showing largest cell and nuclear sizes, as well as highest frequencies of the largest cell size, among all cohorts. The increased PCV, RCC and Hb in males in the summer and autumn (Chapter 2.3.4), were most probably due to increased erythrocyte numbers (RCC), since erythrocytes and nuclei were smaller and more elongate in these drier seasons compared to winter and spring. The increase in erythrocyte size, together with an increase in their numbers, indicates the mechanism responsible for the erythropoietic - stimulating effects of androgens in males (Gardner & Gorshein 1973) during the mating season.

Erythrocytes in females tended to be smaller with smaller nuclei than males, despite females having larger body sizes (see Fig. 2.4). While smallest erythrocytes in adults were more dominant in circulation in summer, in contrast to males, this pattern continues into autumn in females, Since female rubricyte counts were comparably low in autumn, the small size cell class probably represents small, mature erythrocytes. Further supporting this is nuclear shape: through all seasons female nuclei are more circular than males and juveniles, except in autumn, where nuclei are more elongate, characteristic of mature erythrocytes. This could account for the high summer Hb concentrations in females (see chapter 2.3.4), since mature erythrocytes contain more haemoglobin than immature erythrocytes (Bernstein 1938).

Juvenile tortoises showed significant differences in the spring, where erythrocytes and nuclei were larger than in adults, and frequencies of largest erythrocytes in circulation were higher than in adults. This, and higher spring nuclear to cellular

ratios compared to adults, suggests a proliferation of younger erythrocytes in circulation. Since juvenile haemoglobin concentrations are lower than those of adults in the spring (chapter 2.3.4), this further lends evidence that immature erythrocytes have lower haemoglobin concentrations than in mature erythrocytes. These spring differences imply increased metabolic rates in juveniles, which could be attributed to increased growth rates compared to those of adults, which is typical for reptiles (Pienaar 1962) as slower adult growth rates are evident following sexual maturity. While in the summer and autumn, there was evidence for haemoconcentration in adult erythrocytes, juvenile erythrocytes were more circular than in adults, which suggests that juveniles were more resilient to dehydration in the dry seasons, presumably due to a lack of added physiological demands of reproductive pressures.

3.5 CONCLUSIONS

Blood profiles are used in clinical practice to determine an animal's health status. Due to the clear functional role of the erythrocyte in metabolic facilitation, morphological descriptions, including shape, size, colour and irregularities of both erythrocytes and nuclei, are important characteristics and have been described for a few tortoise and turtle species, although knowledge in this field is still lacking for many chelonians. Exacerbating this has been the inconsistent terminologies for the different developmental stages of erythrocytes. This is the first study to describe erythrocyte morphology and development, as well as to investigate seasonal changes in blood profiles among male, female and juvenile geometric tortoises.

Erythrocytes in circulation were represented through the full continuum of developmental stages including immature rubricytes, polychromatophils, mature and senile erythrocytes. Growth in rubricytes was evident by cytoplasm increase, and increased haemoglobin concentrations in the cytoplasm. As the cells matured from the polychromatophil stage, nuclear condensing and cellular and nuclear elongation was observed. Immature erythrocytes proliferated in circulation in winter and spring and this erythropoietic response is related to the limited metabolic activity of ectotherms during colder conditions.

The lack of significant degenerative responses as well as parasites in circulating blood indicates this population of geometric tortoises to be clinically healthy, however, symptoms of abnormalities included erythrocyte inclusion bodies, vacuoles and poikilocytosis, and were more prevalent in the drier autumn months, suggesting

seasonally-based stress conditions. Evidence for haemoconcentration in the dry seasons and haemodilution in the wetter sampling periods result from changes in red blood cell size and shape. Erythrocyte morphometrics are useful in quantifying morphological changes, and together with haematological parameters, further illuminate differences in blood profiles. Changes in nuclear to cellular area ratios corresponded to maturation in erythrocytes, whereas changes in circularity and elongation can indicate cytoplasmic swelling or shrinking; indicative of haemodilution and haemoconcentration respectively.

Erythropoiesis is affected by a host of exogenous and endogenous factors including age, climate, nutrition, reproductive state and disease. Marked seasonal differences in blood profiles highlight the importance of external environmental conditions, acting as cues for the timing of important physiological processes. Cohort differences in geometric tortoises suggest these physiological processes revolve around increased growth rates in juveniles, and reproductive pressures in adults. The erythropoietic response in winter and spring, together with the prevalence of mature erythrocytes during summer and autumn highlight the mechanisms enabling elevated or supressed metabolic functioning and thus activity levels in response to differing climatic conditions.

Being a heterogeneous group of vertebrates, reptilian blood cell morphology is highly varied, and representative values are necessary for the different genera and species. Results obtained in this study could serve as baseline haematological values to monitor the physiological responses of the Critically Endangered geometric tortoise.

4 WHITE BLOOD CELL AND THROMBOCYTE HISTOLOGY

4.1 INTRODUCTION

The complete leukocyte evaluation includes a total white blood cell count, determination of the differential leukocyte count, and a description of leukocyte morphology based on analysis of the blood smear. Laboratory techniques are similar to those used in mammalian studies, although reptilian blood differs from mammalian blood through nucleation of all cells, pluripotentiality of certain cell types and the inevitable direct effect of influences such as gender, age, environmental conditions and nutritional status (Zhang *et al*. 2011). Difficulties in leukocyte descriptions arise from inconsistent terminology and uncertain cellular lineages. Nomenclature has largely been based on the cell's lineage, cytological (including staining) characteristics and the cell's function (Pienaar 1962). In addition to the leukocytes, of which up to seven types have been described in reptiles, the circulating blood also includes nucleated thrombocytes.

Leukocytes can be divided into two major groups; the granulocytes and agranolucytes. The granulocytes include heterophils, eosinophils and basophils. Heterophils replace the mammalian neutrophil in reptiles. The heterophils function against bacterial infections and are relatively large, round cells with clear cytoplasm with many spindle-shaped granules, which generally stain eosinophilic pink-orange or reddish-brown in chelonians (Frye 1991). These cells are described as being fragile, easily distorted in the production of a blood smear, and are known to display chromophobic (weak-staining) properties in 'watery' solutions. The heterophil is described as a Type I eosinophil by Pienaar (1962), being derived from granuloblasts (myeloid stem cells), while immature cells display a relatively larger nucleus and cytoplasm stains more basophilic. Heterophil numbers in circulation display most inconsistency among reptiles, and Jacobson (2007) noted it as the most predominant leukocyte type in Chelonia. In cases of infectious inflammatory disease, toxically reactive cells, in which basophilic or vacuolated intracytoplasmic granulation is observed, are more numerous. In reptiles, highest heterophil numbers occur in the summer months and lowest numbers during hibernation, although Jacobson (2007) also noted a stress/gravidity-related increase.

Eosinophils are morphologically and functionally similar to those in mammals, although it is not described in all reptile species. They are round cells containing distinct spherical granules which stain faint-pink to deep orange-red in the abundant cytoplasm (Frye 1991). A distinction from heterophils is the shape of the cytoplasmic granules, as well as eosinophils appear less distorted in shape (Zhang *et al.* 2011). Pienaar (1962) reported eosinophils as being derived from lymphocytoid cells. Whereas heterophils typically react to extra-cellular bacterial infections, eosinophils react to allergens, parasitic infestations (Frye 1991; Strik *et al.* 2007) and reactive forms may appear degranulated and/or vacuolated.

Basophils, as their mammalian counterparts, are the most readily identifiable granulocyte, being small, round with dark-purple staining, uniformly metachromatic intracytoplasmic granules and a dark nucleus which is often obscured (Zhang *et al.* 2011). Due to their water-sensitivity, basophils may appear degraded, resulting in faint, smudged 'ghost' cells in the blood slide (Strik *et al.* 2007). Basophils typically occur in constant numbers in circulation, with minimal seasonal influences, yet counts among reptile species are very varied. Basophils are described as 'mast' leukocytes and function in inflammation (Davis *et a*l. 2008), histamine release and may be associated with haematoparasites as well as certain viral infections (Strik *et al.* 2007).

The major agranulocytic leukocytes are the lymphocytes and monocytes. Lymphocytes reportedly display diversity beyond T and B cell forms (Campbell 1996), and Pienaar (1962) reported them as the main blood-cell progenitor for most leukocyte types. In the blood smear, they are easily mistaken for thrombocytes, however certain morphological and cytochemical differences between the two are discernable in that lymphocytes tend to be larger cells, with a distinct border and lightly to moderately basophilic cytoplasm, with larger nuclei and a higher nuclear to cytoplasm area ratio than thrombocytes (Strik *et al.* 2007). They are noted to be more numerous in females, and also during warmer periods, possibly due to the relative inability of some temperate species to invest in a primary immune response during low environmental temperatures (Frye 1991). Lymphocytes are associated with inflammation, parasitic infestations as well as antigenic stimulation (Campbell 1996; Canfield 1998), reactive forms have been observed, as well as what Strik *et al.* (2007) refer to as plasmacytoid forms.

Plasma cells represent a specialised lymphocyte in response to a specific antigen. Pienaar (1962) identifies plasma cell-lymphocyte transition stages in the peripheral blood of reptiles. While they resemble lymphocytes, in comparison, plasma cells are less abundant than lymphocytes (Frye 1991) and the cytoplasm stains a deeper blue, with a more intense blue eccentrically-placed nucleus. Plasma cell composition in circulation may increase in cases of severe infections or inflammatory disease (Strik *et al.* 2007).

Monocytes are similar in morphology and function to their mammalian counterparts, often described as large, with a large, kidney-shaped nucleus surrounded by abundant pale-blue staining cytoplasm (Zhang *et al.* 2011). Owing to their largely phagocytic function, reactive monocytes can be seen with ingested intracytoplasmic particles, sometimes referred to as macrophages. Monocytes are associated with chronic infection, inflammation, immunogenic stimulation, as well as bacterial infections (Frye 1991; Campbell 1996; Canfield 1998).

Azurophils have been described in chelonian circulating blood, and have been closely allied to monocytic leukocytes. Frye (1991) refered to them as neutrophils, while they have been described as monocytoid azurophils (Campbell 1996) and as having both monocytic and granulocytic features (Pienaar 1962; Strik *et al*. 2007). In independent haematological studies in the turtle *C. mydas*, Samour *et al*. (1998) observed azurophils in circulation, while Work *et al.* (1998) found no evidence of this cell type in peripheral blood.

Reptile thrombocytes are nucleated, involved in the phagocytosis of bacteria, tissue debris and senescent erythrocytes, and as mammalian platelets, haemostasis. The cells are often described as small, basophilic-staining, ellipsoidal with pale, delicate cytoplasm which can easily be distorted, and a relatively large, distinctly darker basophilic nucleus (Pienaar 1967; Zhang *et al.* 2011). Although often resembling small lymphocytes, a distinguishing characteristic of thrombocytes is the aggregration of the cells on a blood smear. Derived from thromboblasts, these cells (together with erythrocytes) can divide amitotically and have the pluripotentiality to transform into erythrocytes (Frye 1991). As a result, a range of transitional phases can be seen in the circulating blood and due to their phagocytotic role, reactive cells can be seen with pseudopodia and/or cytoplasmic vacuolation (Frye 1991; Canfield 1998; Strik *et al.* 2007).

The objectives of this study were to: (1) identify and describe morphological characteristics of leukocyte types and thrombocytes in peripheral blood of *P. geometricus*; (2) assess differences in the leukocyte and thrombocyte profiles of males, females and juveniles; and (3) evaluate seasonal changes in leukocyte and thrombocyte profiles.

4.2 MATERIALS AND METHODS

4.2.1 Sampling procedure

Blood samples were obtained over four seasons at Elandsberg Nature Reserve (3 800 ha, 33° 26' S; 19° 01' E) in the southwestern Cape, South Africa, from 26 to 42 healthy, free-ranging geometric tortoises (including males, females and juveniles) per season (see Table 2.1 for sample sizes in different seasons). I sampled blood from unanaesthetised tortoises immediately after capture to limit stress-induced changes to blood parameters. The mass of the animals determined the maximum blood volume sampled and I took care not to exceed 0.5% of the animal's field body mass (a conservative veterinary standard). I used a 25 G needle with a 1 or 2 ml syringe to collect blood from either the jugular vein or carotid artery. Since EDTA is known to cause lysis of chelonian cells (Harding *et al*. 2005; Knotek *et al.* 2006), heparin was used as an anticoagulant, although it has been observed to impart a blue tinge to blood smears as well as affect the clumping nature of cells (Houwen 2000; Strik *et al*. 2007). Sampling normally took 1 to 2 minutes and I aborted attempts if an adequate sample has not been obtained in approximately 5 minutes. The animals were kept under observation for 24 hours and during the dry season, I provided access to drinking water before returning the animals to the capture site.

I produced blood smears, in duplicate, using the wedge-smear technique with a single-use, bevel-edged glass slide spreader (Pendl 2006). Smears were air-dried, fixed in absolute methanol, and stored in dust-free boxes until being stained. I obtained best staining results from the May-Grünwald – Giemsa stains, using the technique described in Houwen (2000). To prepare the stock solutions, May-Grünwald reagent powder (0.3 g) was mixed in 100 ml absolute methanol, left to stand overnight, and subsequently filtered. Giemsa reagent powder (1 g) was mixed in 66 ml glycerol, heated to 56 ºC for 100 minutes, mixed with 66 ml absolute methanol, left to stand overnight, filtered and stored in an airtight container. I used a buffer of pH 6.8 to dilute stock solutions each time before staining; May-Grünwald stock was mixed with equal parts of buffer whereas Giemsa stock was diluted with

nine parts buffer. The May-Grünwald stain was introduced to the blood smear, letting stand for 5 minutes, after which the excess solution was drained from the slide that was then introduced to the Giemsa stain for 12 minutes. The slide was then rinsed once with the buffer solution, washed in, and left to stand in distilled water for 3 minutes. Stained blood smears were left to dry and later fitted with a glass cover slip using Entellan New rapid-mounting medium for microscopy (Merck).

4.2.2 Histological evaluation and measurements

I used all the smears to identify, photograph and measure 10 representative cells of each leukocyte type and of thrombocytes. I used a Leica DM 500 photomicroscope (Leica LAS Software, Leica Microsystems Ltd., Switzerland, version 1.8.0), with 10x eyepieces, for the histological evaluation of leukocytes and thrombocytes under immersion oil with a 100x objective to give 1000x magnification. For thrombocytes and each leukocyte type, I assessed the size, shape and staining characteristics of cells and their nuclei for identification and a detailed description of each cell type.

To determine size ranges of leukocytes, thrombocytes and their nuclei, I used a Leica ICC50 camera linked to the Leica DM 500 digital photomicroscope (100x objective and total magnification of 1000x) to take digital images of blood cells. The images were saved as jpeg files (2048 x 1536 pixels) and then analysed using the NIKON NIS Elements imaging software (refer to chapter 3.2.2 for image analysis technique).

Nuclei in basophils were obscured by dark cytoplasmic granules, and so were not measured. For each other leukocyte, I measured the area of both cell and nucleus (surface area of the image in μm^2). Cell measurements were digitally automated to a precision of 0.01 µm. Pixel size was manually calibrated using a micrometer scale automatically generated when capturing the images (at 1000x magnification, 1 pixel = 0.07µm).

In addition, for each individual, I performed a differential white cell count, using the meandering technique to identify the first 100 leukocytes encountered. Leukocytes were counted as heterophils, eosinophils, basophils, lymphocytes, plasma cells, monocytes or azurophils. All thrombocytes encountered within the first 100 leukocytes were counted, and reported as relative to 100 leukocytes. Data were exported to Windows Excel (MS Office) and collated into one spreadsheet for statistical analysis.

4.2.3 Data and statistical analysis

SigmaStat (SPSS Inc., Chicago, U.S.A. version 2.03) was used to statistically evaluate leukocyte and thrombocyte counts. Square root transformations were sufficient for most parameters to comply with parametric requirements, however certain tests still failed normality and/or equal variance. Due to low numbers, I analysed plasma cell and azurophil counts separately. To test for the effects of season and cohort on each individual cell type, I used two-way ANOVAs followed by Student-Newman-Keuls post hoc comparisons. Furthermore, one-way ANOVAs were used to test for differences in cellular and nuclear areas among cell types, following $\log_{10}$ transformations. When data were parametric, one-way ANOVA was followed by Student-Newman-Keuls post hoc comparisons, whereas for non-parametric data, Kruskal-Wallis ANOVA on ranks was followed by Dunn's multiple post hoc comparisons.

4.3 RESULTS

4.3.1 Leukocyte types, thrombocytes and features

Seven leukocyte types were identified circulating in peripheral blood of *P. geometricus* (Fig. 4.1). These included the heterophils, eosinophils, basophils, lymphocytes, plasma cells, monocytes and azurophils. Additionally, the smears contained thrombocytes.

Heterophils were typically round, large cells, ranging between 121.56 and 226.25 μm^2 in surface area and often appeared polymorphic (Fig. 4.2a - b). Nuclei ranged between 29.42 and 52.19 μm^2, and nuclear to cellular ratios ranged from 17.2% to 31.9%. A distinguishing character of the eccentrically-placed heterophil nucleus was the clumping of nuclear material, with darker staining clumped chromatin within paler-staining abundant parachromatin (Fig. 4.2a - c). Sometimes bi-lobed nuclei were observed (Fig. 4.2c). The abundant clear cytoplasm contained dense, spindle-shaped eosinophilic granules which stained faint pink to deep red, and in reactive heterophils, a deep lilac (Fig. 4.2d). Often, heterophils appeared with ingested particles within the cytoplasm (Fig. 4.2e). In some cases, degranulation (Fig. 4.2f) and senescence (Fig. 4.2g) was visible, resulting in eosinophilic cellular debris (Fig. 4.2h).

Eosinophils were distinctly round cells ranging between 59.54 and 145.16 μm^2 in surface area. The slightly basophilic cytoplasm contained distinct spherical eosinophilic globules, which ranged in staining from reddish brown to orange (Fig. 4.3a). The blue nuclei were typically lentiform, often seen displaced to one pole (Fig. 4.3b), and ranged between 15.01 and 45.53 μm^2 in size. Nuclear to cellular ratios ranged from 24.3% to 44.6%. Eosinophils showed less polymorphy compared to heterophils, and the eosinophilic nucleus appeared more homogenous in chromatin and parachromatin texture (Fig. 4.3c). In some cases, degranulated eosinophils were present (Fig. 4.3d).

Basophils were round cells, readily identifiable due to their intense basophilic staining (Fig. 4.4a), and ranged between 53.82 and 135.96 μm^2 in surface area. The cytoplasm was densely packed with round, dark blue-violet staining granules (Fig. 4.4b) that often obscured the dark blue nucleus (Fig. 4.4c). Often seen, were damaged cells, resulting in a burst/leaked appearance, with the hyperchromatic granules released into the blood plasma (Fig. 4.4d).

Lymphocytes ranged from small to large forms (Fig. 4.5a - b), between 68.55 and 147.14 μm^2 in surface area. The cells were round, and nuclei and cytoplasm both stained basophilic blue to grey, often both with a homogenous appearance (Fig. 4.5a), rendering the nuclei difficult to identify. Nuclei ranged between 30.03 and 59.03 μm^2 in surface area, and nuclear to cellular area ratios ranged between 46.2% and 79.2%. Medium-sized lymphocytes were often reactive, in which the cytoplasm appeared vacuolated (Fig. 4.5c - d). Less commonly noted, were small cytoplasmic projections in reactive forms (Fig. 4.5d).

Plasma cells were uncommon in peripheral blood in *P. geometricus*. These basophilic cells were round with large nuclei, which were often difficult to distinguish within the intensely staining, abundant blue cytoplasm (Fig. 4.6). These characteristics bore a close resemblance to lymphocytes. Due to its scarcity, only one cell was measured (Table 4.1), revealing a large nuclear to cellular ratio (46.7%).

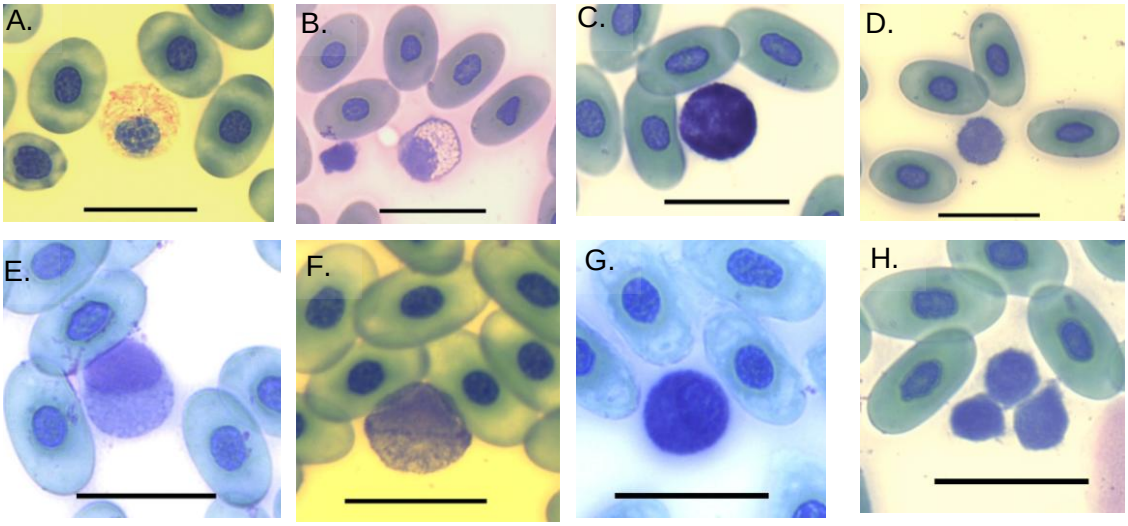

Figure 4.1 Leukocytes in circulating blood of *Psammobates geometricus*, showing the heterophil (a), eosinophil (b), basophil (c), lymphocyte (d), plasma cell (e), monocyte (f) and azurophil (g), as well as thrombocytes (h). Scale represents 20 µm, 1000x magnification, Romanowsky stains.

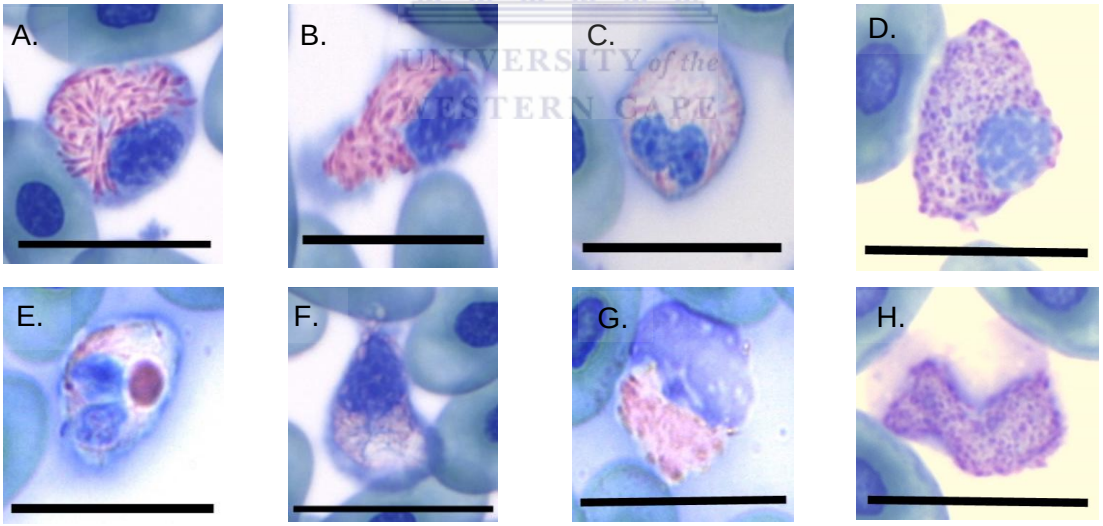

Figure 4.2 Heterophils in circulating blood of *Psammobates geometricus*, showing spindle-shaped cytoplasmic granules, clumping of chromatin (a – b), a bi-lobed nucleus (c), lilac-staining cytoplasmic granules (d), ingested particles (e), degranulation (f), senescence (g) and cellular debris (h). Scale represents 20 µm, 1000x magnification, Romanowsky stains.

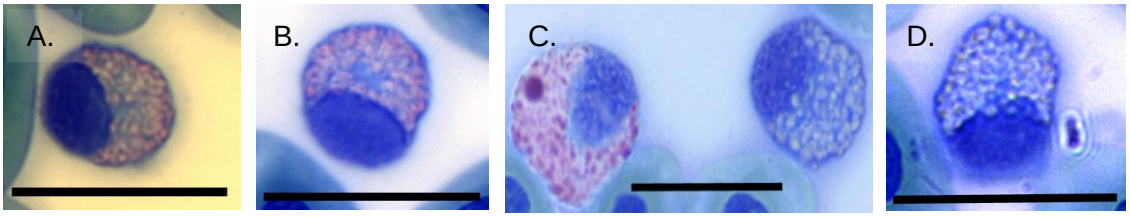

Figure 4.3 Eosinophils in circulating blood of *Psammobates geometricus*, showing spherical cytoplasmic granules (a – b). Note differences to a heterophil (c; heterophil on right) and cytoplasmic degranulation (d). Scale represents 20 µm, 1000x magnification, Romanowsky stains.

Figure 4.4 Basophils in circulating blood of *Psammobates geometricus*, showing intensely basophilic cytoplasm and nuclei (a – c) as well as a ruptured cell (d). Scale represents 20 µm, x1000 magnification, Romanowsky stains.

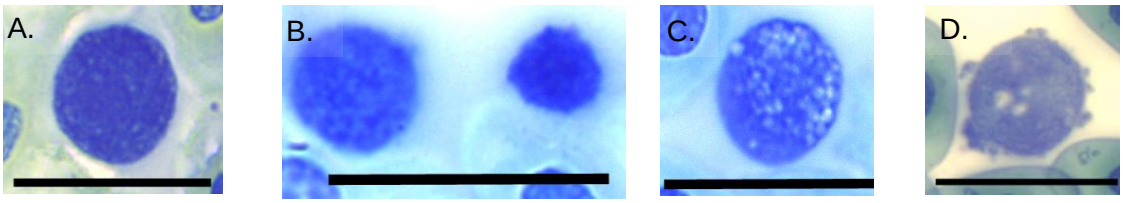

Figure 4.5 Small and medium lymphocytes in circulating blood of *Psammobates geometricus*, showing basophilic cytoplasm and nuclei (a - b), cytoplasmic vacuolation (c), and projections (d). Scale represents 20 µm, 1000x magnification, Romanowsky stains.

Monocytes were typically large (surface areas ranged from 110.02 to 245.95 μm^2), round, basophilic cells which varied in staining reactions from purple, blue or grey (Fig. 4.7a - c). The nuclei were very large (ranging between 51.54 and 151.54 μm^2), generally amorphous, and displaced the abundant cytoplasm, which tended to be paler in staining intensity (hypochromatic; Fig. 4.7a - c). Nuclear to cellular ratios were between 35.4% and 62.9%. A common feature of reactive cells, was vacuolation of cytoplasm (Fig. 4.7c), while azurophilic granulation has been observed (Fig. 4.7d).

Azurophils were infrequent, identifiable by their distinctly-postitioned nuclei, which were often displaced by the abundant cytoplasm. These cells showed a characteristic purple-staining quality, with the large nucleus staining more intensely than the cytoplasm (Fig. 4.8). Nuclear material was difficult to discern, while the cytoplasm appeared finely granulated. Cellular and nuclear characteristics were similar to those in monocytes. Due to its scarcity, only one cell was measured (Table 4.1), displaying a nuclear to cellular ratio of 35.3%.

Thrombocytes were numerous in the blood smear, ranging in size between 25.44 and 47.91 μm^2, generally appearing as round to oval (Fig. 4.9a), although the cells were often irregularly shaped. Thrombocytes were identifiable by a prominent, often intensely blue staining, irregularly-shaped nucleus (ranging between 20.67 and 35.44 μm^2) surrounded by scant, faintly-staining basophilic cytoplasm (Fig. 4.9b), which was rarely visible intact (Fig. 4.9c). Nuclear to cellular ratios were between 70.9% and 91.4%. A characteristic feature of this cell type was that of forming aggregations (Fig. 4.9a and d), which was useful in distinguishing cellular and nuclear characteristics of thrombocytes from small lymphocytes (Fig. 4.9d). Lymphocytes tended to have more regular cellular membranes, whereas in thrombocytes, the cytoplasm appeared faint, and ruptured easily. Lymphocyte nuclei were difficult to discern from cytoplasm, whereas thrombocyte nuclei tended to be more easily identifiable, and irregular in shape.

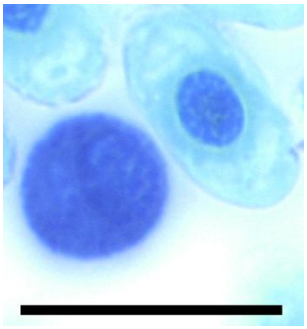

Figure 4.6 Plasma cell in circulating blood of *Psammobates geometricus*, showing intensely blue-staining cytoplasm and large nucleus. Scale represents 20 µm, 1000x magnification, Romanowsky stains.

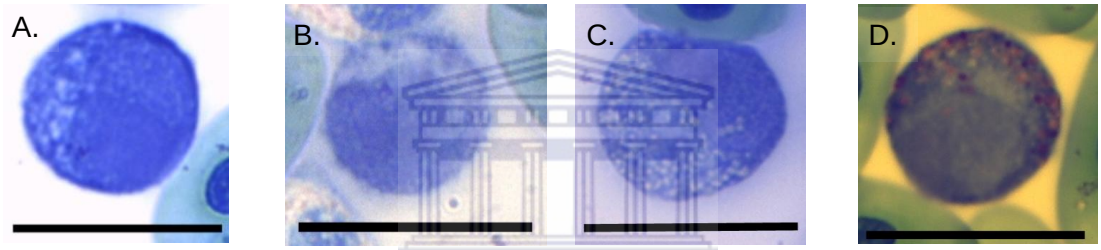

Figure 4.7 Monocytes in circulating blood of *Psammobates geometricus*, showing hypochromatic cytoplasm, large nuclei (a – c), cytoplasmic vacuolation (c) and azurophilia (d). Scale represents 20 µm, 1000x magnification, Romanowsky stains.

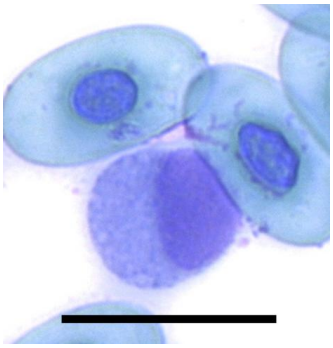

Figure 4.8 Azurophil in circulating blood of *Psammobates geometricus*, showing characteristic purple-staining, abundant cytoplasm and large nucleus. Scale represents 20 µm, 1000x magnification, Romanowsky stains.

Other cell types observed in the leukogram included small, round, possibly immature granulocytes (heterophils or eosinophils) showing relatively large nuclei and cytoplasmic basophilia (Fig. 4.10a - b). Other types included round, often small, basophilic cells that appeared to be lymphocyte-monocyte transition type leukocytes, showing relatively large nuclei and basophilic cytoplasm (Fig. 4.10c - d). Other varieties of basophilic leukocytes appeared degranulated, vacuolated or both, which rendered them indiscernable (Fig. 4.10e - f).

Monocytes and heterophils had larger surface areas than the other leukocytes had and eosinophils were larger than lymphocytes. All leukocytes were larger than the thrombocytes (Table 4.1, $F_{5,54}$ = 49.99, P < 0.0001). Nuclei were not measured in basophils, due to intensely metachromatic staining in cellular granules. Monocyte nuclei were larger than the nuclei of other leukocytes. Lymphocytes had larger nuclei than eosinophils, and both lymphocyte and heterophil nuclei were larger than in thrombocytes ($F_{4,45}$ = 23.59, P < 0.0001). Nuclear to cellular area ratios were highest in thrombocytes, followed by lymphocytes, monocytes, eosinophils and least in heterophils ($F_{4,45}$ = 91.76, P < 0.0001).

Table 4.1 Cellular and nuclear sizes of circulating leukocytes and thrombocytes in *Psammobates geometricus*, as well as nuclear to cellular surface area ratios. Data are presented as mean and standard deviations of ten cells of each cell type, except for the plasma cell and azurophil (one only).

Cell type	Cellular surface area (µm^2)	Nuclear surface area (µm^2)	N/C ratios (%)
Heterophil	164.80 ± 33.16	39.37 ± 7.15	24.39 ± 4.83
Eosinophil	106.40 ± 31.47	35.85 ± 9.40	34.33 ± 6.51
Basophil	91.56 ± 26.79	-	-
Lymphocyte	76.94 ± 15.04	46.74 ± 7.65	61.72 ± 10.33
Monocyte	170.13 ± 50.50	84.60 ± 32.10	49.22 ± 7.40
Plasma Cell	151.3	70.49	46.66
Azurophil	101.26	35.72	35.28
Thrombocyte	35.46 ± 6.06	28.47 ± 4.33	80.61 ± 6.47

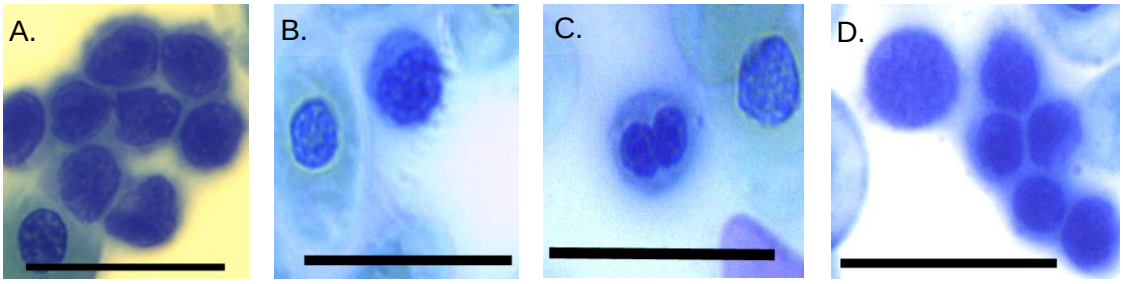

Figure 4.9 Thrombocytes in circulating blood of *Psammobates geometricus*, showing irregularly-shaped nuclei (a), scant cytoplasm (b), bi-nucleation (c) and aggregations (a and d), with a lymphocyte (d). Scale represents 20 µm, 1000x magnification, Romanowsky stains.

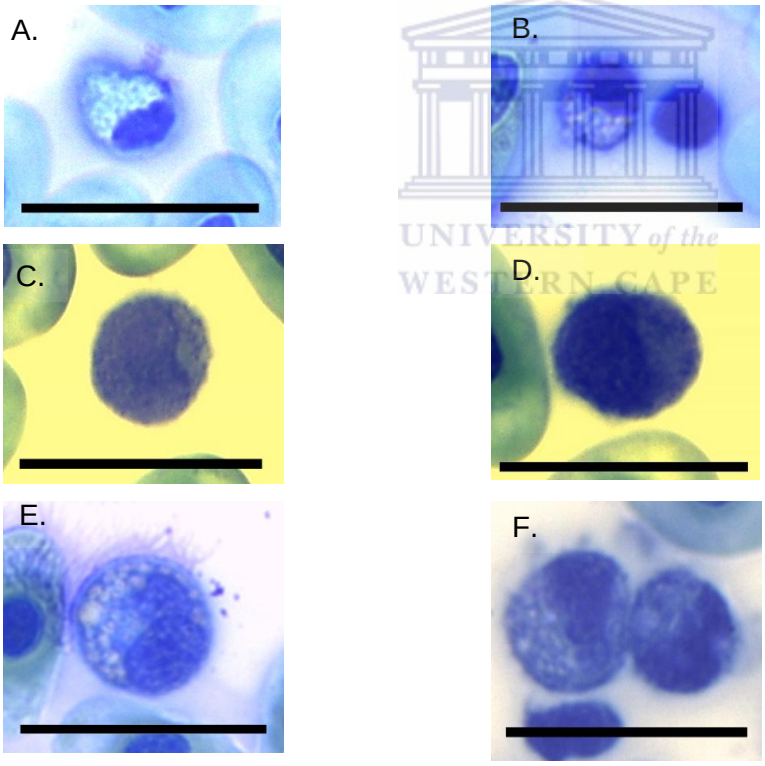

Figure 4.10 Leukocytes in circulating blood of *Psammobates geometricus*, showing immature granulocytes (a - b), mono-lympho transition type leukocytes (c - d), and vacuolated/degranulated leukocytes (e - f). Note the small lymphocyte in b. Scale represents 20 µm, 1000x magnification, Romanowsky stains.

4.3.2 Leukocyte and thrombocyte frequencies

Differential leukocyte counts were made by recording the frequency of each leukocyte type relative to 100 white blood cells whereas thrombocyte counts were recorded per 100 leukocytes (Table 4.2).

Table 4.2 Seasonal differences in differential leukocyte counts, and thrombocyte numbers relative to 100 leukocytes, for cohorts of *Psammobates geometricus*. Data are presented as means and standard deviations.

		Females	Males	Juveniles
Spring	Heterophils (%)	49.40 ± 17.11	56.91 ± 12.23	65.40 ± 13.96
	Eosinophils (%)	6.47 ± 5.67	5.82 ± 3.49	0.40 ± 0.55
	Basophils (%)	3.87 ± 2.64	3.55 ± 2.02	1.20 ± 1.64
	Lymphocytes (%)	36.27 ± 15.42	30.82 ± 9.47	30.60 ± 13.20
	Monocytes (%)	3.93 ± 2.22	2.64 ± 2.77	1.20 ± 1.30
	Plasma Cells (%)	0.07 ± 0.26	0.09 ± 0.30	0.80 ± 1.30
	Azurophils (%)	0.0	0.18 ± 0.60	0.40 ± 0.55
	Thrombocytes	83.60 ± 44.04	112.64 ± 34.41	79.80 ± 18.40
Summer	Heterophils (%)	51.36 ± 14.32	44.00 ± 11.08	38.00 ± 9.33
	Eosinophils (%)	11.91 ± 7.85	16.25 ± 12.45	8.00 ± 15.10
	Basophils (%)	3.64 ± 2.84	3.88 ± 3.48	3.14 ± 1.57
	Lymphocytes (%)	29.73 ± 12.91	33.50 ± 11.49	45.43 ± 8.98
	Monocytes (%)	3.18 ± 4.12	2.00 ± 3.78	4.43 ± 3.95
	Plasma Cells (%)	0.18 ± 0.40	0.25 ± 0.71	0.86 ± 1.07
	Azurophils (%)	0.0	0.13 ± 0.35	0.14 ± 0.38
	Thrombocytes	137.73 ± 26.87	140.25 ± 28.26	160.71 ± 41.98
Autumn	Heterophils (%)	46.70 ± 21.33	48.17 ± 11.57	41.00 ± 14.73
	Eosinophils (%)	8.50 ± 7.75	8.67 ± 7.08	3.20 ± 3.56
	Basophils (%)	4.10 ± 6.17	2.75 ± 3.25	2.80 ± 2.17
	Lymphocytes (%)	37.80 ± 23.74	37.42 ± 15.10	48.20 ± 18.27
	Monocytes (%)	2.30 ± 2.06	2.75 ± 1.91	3.80 ± 4.82
	Plasma Cells (%)	0.30 ± 0.48	0.17 ± 0.39	0.80 ± 1.30
	Azurophils (%)	0.30 ± 0.67	0.08 ± 0.29	0.20 ± 0.45
	Thrombocytes	106.30 ± 49.07	118.83 ± 42.04	130.40 ± 48.92
Winter	Heterophils (%)	57.75 ± 7,78	51.38 ± 10.13	55.46 ± 16.26
	Eosinophils (%)	8.13 ± 4.08	11.31 ± 6.85	2.38 ± 2.57
	Basophils (%)	5.56 ± 10.31	1.62 ± 2.10	3.85 ± 3.56
	Lymphocytes (%)	26.25 ± 14.14	32.15 ± 16.04	35.46 ± 15.77
	Monocytes (%)	1.25 ± 1.29	2.00 ± 1.91	1.69 ± 2.53
	Plasma Cells (%)	1.06 ± 1.24	1.54 ± 2.40	1.15 ± 2.41
	Azurophils (%)	0.0	0.0	0.0
	Thrombocytes	111.25 ± 48.32	136.92 ± 38.40	136.15 ± 65.90

4.3.3 Effects of season and cohort

Because plasma cells and azurophils had low representation in differential white cell counts, I excluded these cells in order to do two-way ANOVAs to evaluate the effects of season, cohort and type. Within the cohorts, there were no seasonal effects on differential white cell counts ($P > 0.183$). In adult geometric tortoises, the pattern of leukocyte type frequencies was the same; heterophils were most abundant, followed by lymphocytes, then eosinophils, with no difference between monocyte and basophil counts ($F_{4,\ 200\ or\ 240} > 210.2$, $P < 0.0001$). Males showed an interaction of season and leukocyte type ($F_{12,200} = 2.21$, $P = 0.013$), and the general pattern applied in autumn and winter, whereas in summer, heterophils and lymphocytes were equally abundant.

In spring, eosinophil counts were the same as monocytes and basophils. Additionally, male eosinophil values were higher in summer than in spring and autumn. In juveniles, the pattern was slightly different; heterophils were most abundant, followed by lymphocytes, but eosinophils, monocytes and basophils did not differ ($F_{4,130} = 178.2$, $P < 0.0001$). Due to an interaction of season and type for juveniles ($F_{12,130} = 2.51$, $P = 0.0053$), the overall pattern appeared in winter and spring whereas in summer and autumn, lymphocyte counts were the same as heterophils. Additionally, heterophil values were higher in spring and winter than in summer, and spring values were also higher than in autumn. Furthermore, eosinophil counts were higher in summer than in spring.

Within season analyses showed a cohort effect in spring ($F_{2,140} = 3.82$, $P = 0.024$) but not in autumn, summer or winter ($P > 0.59$). Furthermore, the frequency of leukocyte types differed within each season ($F > 98.6$, df1 = 4, df2 = 115, 120, 140 or 195; $P < 0.0001$). Leukocyte counts showed similar patterns in winter and summer; most abundant were heterophils, followed by lymphocytes, eosinophils, monocytes and basophils, with no difference between the latter two types. Autumn lymphocyte and heterophil counts did not differ, with no interactions ($P = 0.580$). Winter tests showed an interaction of cohort and cell type ($F_{8,195} = 4.36$, $P < 0.0001$) and revealed that males displayed the overall pattern, whereas in females, basophils were more abundant than monocytes, while in juveniles, eosinophil counts were not higher than in monocytes and basophils. Additionally in winter, adult values for eosinophils were higher than juvenile values. In summer, heterophils were most abundant, followed by lymphocytes, eosinophils, with monocytes and basophils equally least abundant, with an interaction of cohort and cell type ($F_{8,115} = 3.06$, $P = 0.0037$). This pattern applied to males only, because in females there was no difference between eosinophil,

basophil and monocyte numbers, and in juveniles, lymphocyte counts were the same as heterophils, and eosinophil, monocyte and basophil counts did not differ. In summer, heterophils were more abundant in females than in juveniles, and lymphocytes were more abundant in juveniles than in adults. In spring, there was an interaction between cohort and cell type ($F_{8,140}$ = 2.64, P = 0.010). Heterophils were most abundant, followed by lymphocytes, with no difference among eosinophil, basophil and monocyte counts for each cohort. Heterophil, lymphocyte, basophil and monocyte counts were the same in all cohorts, whereas eosinophils were more abundant in adults than in juveniles.

Because plasma cells and azurophils had such low occurrences (0% to 2% for all cohorts among all seasons), it was not possible to do two-way ANOVAs on these cell types. Instead, I have done one-way ANOVAs on cohort within each season, and on season within each cohort. The differential frequency of plasma cells did not differ among cohorts or among seasons (P > 0.0089 did not meet Bonferroni corrected P-value of 0.0071). Similarly, azurophil differential frequencies did not differ among cohorts or among seasons (P > 0.035 did not meet Bonferroni corrected P-value of 0.0071).

Thrombocyte counts (relative to 100 leukocytes) ranged between 113 and 140 cells in males, 84 and 138 cells in females, and 80 and 161 cells in juveniles. In spring, counts were between 80 and 113, in summer between 138 and 161, autumn counts were between 106 and 130, and in winter, between 111 and 137 cells per 100 leukocytes. Thrombocyte counts changed seasonally ($F_{3,114}$ = 6.79, P = 0.00030), but not with cohort (P = 0.110), and with no interactions (P = 0.759). Summer, winter and autumn counts were higher than in spring.

4.4 DISCUSSION

The complete leukogram includes determination of the differential leukocyte count, thrombocyte count and evaluation of the overall cellular morphology. The classification criteria of chelonian leukocytes vary among studies, as some cells are not easily identified based on their morphological differences alone, and cell lineages are unclear.

Variation in nomenclature and classification is less common in recent literature. Saint Girons (1970) described eosinophils, azurophils, neutrophils and plasma cells in

reptiles. Bernstein (1938) described neutrophils, Pienaar (1962) described these as Type I eosinophils, while recent agreement is that reptilian heterophils are analogous to mammalian neutrophils (Frye 1991; Strik *et al*. 2007). Bernstein (1938) also included the leukocyte type macrocytes, in which his description of this cell type is similar to those for monocytes. Lineages of certain leukocytes are also unclear. Heterophils and eosinophils (commonly referred to as acidophils) have been regarded as one cell in different stages of development (Zhang *et al*. 2011), however Knotkova *et al*. (2002) and most authors report them as separate cell types. Frye (1991) reported the pluripotentiality of thrombocytes into erythrocytes, while Pienaar (1962) described medium-sized lymphocytes as being the chief leukocyte progenitor, and small lymphocytes giving rise to thrombocytes.

In addition to the nucleated thrombocytes, seven leukocyte types were identified in circulating blood of *Psammobates geometricus.* These include the heterophils, eosinophils, basophils plasma cells, lymphocytes, monocytes and azurophils. Leukocytes and thrombocytes were similar to those described in reptiles and other chelonian species, with variation among cell type characteristics, percentage composition, as well as seasonal and cohort differences. Leukocyte profiles reveal the relative white blood cell composition in circulation at the time of sampling and alone can not infer immunocompetence. At a population level, increased numbers of leukocyte types can indicate a general response to a common stimulant / stressor (Davis *et al.* 2008).

Heterophil counts ranged between 38% and 65%, and heterophils were the most abundant leukocyte in males and females, and juveniles. Strik *et al*. (2007) and Sykes *et al.* (2008) report them as the predominant leukocyte type in chelonians and reptiles respectively, Pienaar (1962) reported Type I eosinophils as ranging between 13% and 15%, Frye (1991) reports counts between 30% and 45% in healthy reptiles, and generally, numbers are known to show variation within individuals and species. This is attributible to morphological variations due to a possible chromophobic reaction in watery solutions (for example in Giemsa reagents; Pienaar 1962) as well as the cell maturing in circulation (Sykes *et al.* 2008). Immature heterophils were noted in *P. geometricus*, which were smaller, stained more basophilic, with larger nuclei than in mature heterophils. Reactive forms were observed in which cytoplasmic vacuolation, degranulation and/or basophilia was evident. The primary function of heterophils is phagocytosis, responding mainly to bacterial infections (Frye 1991; Canfield 1998; Strik *et al*. 2007). Although Campbell (1996) reported an

increase in heterophils during the warmer months, in *P. geometricus* spring counts were higher in summer and autumn. Female counts in summer were higher than in juveniles, which could reflect a reproduction-related stress, as described by Strik *et al.* (2007). Heterophil sizes ranged between 122 and 226 μm^2, compared to *Testudo graeca* (Kassab *et al*. 2009), with ranges between 139 and 195 μm^2.

Eosinophils ranged between 0.4% and 16% composition in circulating blood, and were the third most abundant leukocyte. Frye (1991) reports them as comprising 7% to 20% the total leukocyte count in healthy reptiles. Compared to heterophils, eosinophils appeared less easily ruptured, with less polymorphic tendencies. Reactive forms were observed in circulation, in which cells showed an increase in cytoplasmic degranulation and/or basophilia. As in heterophils, immature forms were also observed, being smaller, more basophilic and with larger nuclei than in mature eosinophils. Like heterophils, they function in inflammation, however react primarily to parasitic infestations (ecto-parasites and blood parasites; Frye 1991; Canfield 1998). While large and small forms are not commonly reported in reptiles, Work *et al.* (1998) postulated that in *C. mydas*, large eosinophils were reactive cells in response to parasitic infections or inflammation. Campbell (1996) described reptilian eosinophils as being more abundant in winter and less so in summer, however, in *P. geometricus*, were found to be low in spring for all cohorts. High summer counts in males could indicate an increased immune response during the mating season, since female and juvenile summer eosinophil counts were as low as in monocytes and basophils. Additionally, low juvenile spring and winter eosinophil counts compared to adults may represent a reduced immunological reaction. Eosinophil sizes in *P. geometricus* were between 60 and 145 μm^2, while in *T. graeca* (Kassab *et al*. 2009), ranged between 108 and 132 μm^2.

Basophil counts were low in *P. geometricus*, ranging from 1% to 6% in circulation, while Frye (1991) reports counts between 10% and 25% in normal reptiles. Commonly observed in *P. geometricus*, were ruptured basophils, a condition reported by Pienaar (1962) and Strik *et al*. (2007) in which the cells are described as being easily destroyed by watery solutions. While the function of the basophil is not clearly defined, it is associated with blood parasites and viral infestations, and is involved in immune responses (Canfield 1998; Strik *et al*. 2007). Campbell (1996) reported varied sizes, numbers, and minimal seasonal influences. Frye (1991) reports basophil numbers increasing in the active seasons, and decreasing during hibernation. While in *P. geometricus,* basophil counts were consistently as low as in

monocytes, in females, winter basophil counts were higher than in monocytes, which likely represented an increased immunological response during gravidity. Basophil sizes were between 54 and 136 µm^2, while in *T. graeca* (Kassab *et al*. 2009), ranged between 108 and 103 µm^2.

Lymphocyte counts ranged between 26% and 48%, and were the second most prolific leukocyte in *P. geometricus*. Common to reptilian blood, small, medium and large forms were observed, smaller forms being more abundant. Reactive forms were also noted, more commonly in medium-sized lymphocytes, in which cytoplasmic vacuolation and/or inclusions were present. Possible transitional forms between lymphocytes and plasma cells were observed in circulation, as reported by Strik *et al*. (2007). Lymphocytes are associated with inflammation and parasitic infestations (Campbell 1996; Canfield 1998). Lymphocyte counts in *P. geometricus* were higher in autumn, and may have been associated with nutrition and dehydration stress during the dry season. Pienaar (1962) reported lymphocyte counts being higher in juveniles than in adults, and in *P. geometricus*, this was true in summer. Lymphocyte sizes ranged between 55 and 104 µm^2, compared to *T. graeca* (Kassab *et al*. 2009), in which ranges were reported between 48 and 85 µm^2.

Plasma cells were rare, comprising between 0 and 2% of leukocyte composition in circulating peripheral blood. They appeared as round, blue-staining cells with round nuclei in abundant cytoplasm, and nuclei stained more intensely than cytoplasm. They appeared lymphocytic, as described by many authors (Pienaar 1962; Campbell 1996; Canfield 1998). Frye (1991), described them comprising 0.2 – 0.5% leukogram composition in healthy reptiles, and they are believed to increase in cases of severe infections or inflammatory disease (Strik *et al*. 2007).

Monocyte counts were low, ranging from 1% to 4%, while ranges have been reported from 0.5% by Frye (1991), to 10% (Strik *et al*. 2007), while Pienaar (1962) reports values as high as 20%. Reactive forms were observed, in which ingested particles, and/or cytoplasmic vacuolation was present. Monocytes are stimulated by immunogenic pathogens, chronic infections (Frye 1991), and have a microbicidal role in reptiles (Canfield 1998). Monocyte counts are reported to experience little seasonal variation (Pienaar 1962; Campbell 1996) and this was found to apply to *P. geometricus*. Monocyte sizes ranged between 110 and 246 µm^2, while in *T. graeca*, between 100 and 148 µm^2 (Kassab *et al*. 2009).

Azurophils in *P. geometricus* were rarely observed, occupying between 0 and 0.4% of the leukogram composition, identifiable by their characteristic purple-staining affinity, and distinct nuclei. The cytoplasm appeared finely granulated, while the large nucleus resembled that of a monocyte. These cells are reportedly common in snakes and crocodiles (Strik *et al*. 2007), and have been occasionally reported in chelonians. No seasonal or cohort differences were noted. Having been commonly ascribed to both granulocyte and monocyte leukocyte lineages (Knotkova *et al.* 2002; Strik *et al.* 2007), these cells have been described as azurophilic monocytes in reptiles (Campbell 1996) and in *G. agassizii* (Alleman *et al.* 1992).

Thrombocytes in *P. geometricus* were reported between 80 and 160 cells per 100 leukocytes, while Pienaar (1962) reported ranges between 25 and 350 cells per 100 leukocytes in healthy reptiles. Hajkova *et al.* (2000) identified two thrombocyte types in *Agrionemys horsfieldii*, one oval with clear cytoplasm, and one rectangular with basophilic projections, yet in a 2010 study on *A. horsfieldii*, Shadkhast *et al*. describe thrombocytes as elliptical cells with eliptical nuclei. Reactive forms, showing cytoplasmic vacuolation and/or pseudopods (Strik *et al*. 2007) were not observed in *P. geometricus*. The role of thrombocytes is mainly the phagocytosis of senescent erythrocytes and leukocytes, as well as bacteria and tissue debris (Frye 1991; Strik *et al*. 2007). Geometric tortoise spring counts were lowest for all cohort, and may be associated with low erythrocyte senescence, suggesting homeostatic regulation. Thrombocytes ranged in size between 29 and 48 μm^2, and in *T. graeca* (Kassab *et al*. 2009), between 20 and 26 μm^2.

A critical aspect of accurate leukocyte counts is the differentiation between small lymphocytes and thrombocytes (Shadkhast *et al*. 2010). Thrombocytes in *P. geometricus*, in addition to their aggregative nature, had irregular-shaped nuclei and faintly-stained, scant cytoplasm. Compared to thrombocytes, lymphocytes appeared with more of a distinct cellular border, tended to be rounder in shape, and the cytoplasm more basophilic.

Factors affecting variation in leukocyte profiles range from the preparation of the blood smears, to methods and classification criteria used. The use of heparin as an anticoagulant in chelonians has been noted to impart a blue intensity in Romanowsky stains (Houwen 2000) as well as influence aggregation of leukocytes and thrombocytes on the blood smear. Tavares-Dias *et al.* (2008) reported an absence in blood cell aggregation when heparin was diluted with sodium chloride. Leukocyte

classification and leukocyte counts are subjective to cellular clumping, as well as differentiation of thrombocytes from lymphocytes. These factors render automated haematological counts unreliable, and using light microscopy, provide sources of biases when performing estimated and differential leukocyte counts (Tavares-Dias *et al.* 2008).

Assessments of leukogram profiles have proved an important tool in determining health parameters in a number of chelonian species (Harding *et al*. 2005; Knotek *et al*. 2006). Leukocyte profiles have recently been used as an indicator of stress in ecological studies of wild animals, since changes in stress hormones have been shown to cause changes in leukocyte numbers, most notably heterophils, lymphocytes and eosinophils (Davis *et al*. 2008). Conditions of heterophilia and lymphopenia (high heterophil and low lymphocyte numbers) as well as low eosinophil counts have been used to validate stress conditions in vertebrates, although little research has been done in reptiles. To this end baseline reference values are needed, against which to interpret normal or abnormal physiological changes which may be represented in blood profiles. Results obtained from this study provide morphological characteristics of leukocyte types and thrombocytes in peripheral blood of *P. geometricus.* Causes for seasonal and cohort differences in leukocyte profiles are difficult to isolate, and interactions between the two were evident. This further highlights the differing responses to environmental changes among males, females and juveniles, and are most likely explained by reproductive mechanisms. The immunological response of all cohort through periods of both limited and increased metabolic activity, suggested this population of geometric tortoises to be in clinically healthy condition.

4.5 CONCLUSIONS

Evaluation of white blood cell and thrombocyte morphologies and profiles have been used as successful tools in determining the health status of a variety of chelonian species. In this study, thrombocytes as well as seven leukocytes in *Psammobates geometricus* have been described. These included the heterophil, eosinophil, basophil, lymphocyte, plasma cell, monocyte and azurophil, and were similar as described in other chelonian species. Cell size ranges, counts, as well as seasonal profiles of males, females and juveniles were determined, to serve as baseline reference values for this clinically healthy population.

Heterophils and lymphocytes comprised the major leukocyte components, followed by eosinophils, while monocytes and basophils appeared in equally low frequencies; plasma cells and azurophils were rare. It is difficult to isolate sources of variation in leukocyte profiles, yet changes in the population could indicate a response to environmental changes / stresses. Heterophil counts in *P. geometricus* tended to be higher in spring than in summer and autumn, which suggested increased phagocytic activity, while females showed more consistency in heterophil and lymphocyte numbers. This could be associated with continual physical demands of egg production in females, while high summer heterophil counts compared to juveniles, could be related with follicular enlargement. Similarly, the increased basophil count in females in winter suggested an elevated immunological response during gravid stages.

Eosinophil numbers followed the reverse pattern, showing lower numbers in spring for all cohorts. Since both heterophils and eosinophils are associated with immune responses, this could reflect a granulocyte-specific response to differing antigenic stimulants during the summer and spring. It is possible that during the dry season, low body conditions render geometric tortoises more susceptible to external parasitic infestations, since eosinophils are associated with ecto-parasitic infections.

High lymphocyte numbers in autumn were probably associated with nutrition and dehydration stresses typical of the dry season. Lymphocytes in summer were more abundant in juveniles than in adults, which could suggest an increased immune response in the absence of reproductive demands. This was supported by low eosinophil counts in juveniles in winter and spring, compared to adults.

Considering that a major function of thrombocytes is phagocytosis of senescent erythrocytes, low thrombocyte counts in spring most probably reflected the low occurrence of erythrocyte senescence. As there is evidence for erythropoiesis occurring in spring, low thrombocyte numbers could illuminate the haemostasis mechanism in geometric tortoises.

Factors influencing leukocyte profiles include method of blood slide preparation, classification criteria and accurate cellular identification. In addition to this, is the direct effect of environmental factors that influence tortoise physiology, and as such, white blood cell profiles should assess cohorts separately, and seasonal variations

should be considered. To ensure that baseline value ranges are meaningful, researchers should maintain standardised methods of laboratory and analytical technique, as well as consider the important role of the environmental and individual circumstances on leukocyte profiles.

5 GENERAL CONCLUSIONS

Owing to their ectothermal nature, reptiles are inevitably reliant on environmental conditions to regulate important physiological processes to survive. In response to a climate experiencing seasonal fluctuations of cool, wet periods with warm, dry ones, Natural Selection should optimise the survival strategies of terrestrial tortoises during favourable conditions (Henen *et al*. 1998; Christopher *et al*. 1999; Dickinson *et al*. 2002). This involves investing in growth and / or reproductive costs, instigated by seasonal cues involving changes in temperature, rainfall and food availability (Kuchling 1999).

These physiological mechanisms are often reflected in the blood profiles, considering the roles of the red and white blood cells. Erythrocytes circulate haemoglobin, which permits aerobic respiration at the tissues, enabling metabolic functioning and thus physical activity (Hartman & Lessler 1964; Dessauer 1970). The leukocyte types are all involved in producing and maintaining an immune response, while thrombocytes function in haemostasis (Work *et al*. 1998; Sykes & Klaphake 2008). Haematology has been used as a successful diagnostic tool to reflect the health, nutritional and reproductive status in a number of chelonian species, as well as understand the physiological responses to environmental changes.

Environmental changes in temperature, rainfall and food supply are reflected in seasonal differences in circulating erythrocyte numbers, size, morphology, composition (Frair 1977; Anderson *et al.* 1997; Zhang *et al*. 2011) as well as leukocyte numbers and morphology (Campbell 1996; Canfield 1998; Strik *et al*. 2007). During the winter and spring, low temperatures cause a reduction in metabolic rate (Kuchling 1999), and in *P. geometricus*, was reflected by low haemoglobin and mean cell haemoglobin concentrations. Winter rainfall likely caused hydration states, involving an increase in blood plasma and haemodilution. This is supported by the proliferation of the largest erythrocyte size class coupled with high cellular circularities. The abundance of preferred food supply following rainfall (Balsamo *et al*. 2005; Joshua *et al.* 2005) most probably induced an erythropoietic response, evident by the abundance of immature erythrocytes in circulation. This regenerative response was presumably in preparation for increased metabolic rates associated with increased foraging and mating behaviour typical of the following seasonal cycle (Hofmeyr & Henen, unpublished data). Leukocyte profiles suggested an active immune response during the cooler periods. Heterophils were more abundant in the

spring, while eosinophils were relatively low, compared to other seasons. This suggested a granulocyte-specific immune response to different stimulants / stresses.

In the dry summer and autumn, higher temperatures enabled an increase in metabolic rates, supported by high mean cell haemoglobin concentrations as well as the elliptical shape of erythrocytes, which both suggested increased oxygen-exchange efficiency (Hartman & Lessler 1964). Increased metabolic ability was necessary to engage in mating behaviour and increased foraging for less abundant and less nutritional food supply, both of which occur in the dry season (Baard 1995; Boycott & Bourquin 2000). Reduced water availability likely resulted in haemoconcentration, and was evident through the proliferation of small erythrocytes in circulation. This dehydration stress was also reflected through increased degenerative abnormalities such as senescence and poikilocytosis. Coupled with this, is limited food supply in these dry seasons, and was reflected through low body conditions during autumn. Low body condition, together with the relative abundance of lymphocytes, could suggest a compromised immune defence in the summer and autumn.

Owing to differing growth and reproductive strategies, these physiological responses to seasonal fluctuations affect each cohort differently. In males, haematological changes were most pronounced in summer and autumn; geometric tortoise mating season. Males experience elevated metabolic demands associated with mate-seeking and male to male aggression, and the high packed cell volume, haemoglobin concentration and red blood cell counts were attributed to the erythropoietic effects of male hormones (Gardner & Gorshein 1973; Zitzmann & Nieschlag 2004). The increase in size and abundance of large red blood cells suggested the mechanism by which androgens influence erythropoiesis in males. Elevated eosinophil counts in summer might also indicate an increased immune response, possibly parasite load during mating season.

Female reproductive cycles are more complex, involving ovulation, vitellogenesis and nesting, and are timed to environmental cues throughout the year (Kuchling 1999; Loehr *et al*. 2004). It is likely that erythropoiesis in female geometric tortoises is necessary to facilitate the extra metabolic requirements of foraging activities to satisfy higher nutritional demands associated with larger body size and egg production. High haemoglobin concentrations and mean cell volumes in spring may provide evidence for this, as vitellogenesis occurs in spring with rapid enlargement of

follicles in autumn, in preparation for ovulation after the first autumn rains (Hofmery & Henen, unpublished data). The increased mean cell volume again in autumn suggested that females may experience a second phase of erythropoiesis to accommodate their reproductive requirements. Female heterophil and lymphocyte counts showed greater consistency than in males or juveniles, which may result from continual reproductive requirements. Higher summer heterophil counts compared to juveniles may indicate a gravidity-related stress, while the increased winter basophil counts in females suggested elevated immune responses during the gravid phase.

The lack of reproductive pressures in juveniles allows more energy investment towards growth, and as a result, growth rates slow considerably after sexual maturity has been reached (Pienaar 1962; Baard 1995). There is little evidence for this in *P. geometricus*, as juveniles showed only a difference in haemoglobin concentrations; being lower than in adults. The lack of differences in packed cell volume and red blood cell count could be attributed to a species-specific response to environmental conditions or sample size. Juveniles in spring showed an increase in immature erythrocytes, suggesting an erythropoietic response which is likely to maximise foraging opportunities during favourable conditions. Evidence for haemoconcentration in the drier seasons was less than in adults, since erythrocytes were more circular than in adults, which suggested a greater resilience to dehydration conditions than adults. Lymphocytes were reported to be more abundant in juveniles than in adults, and this applied to *P. geometricus* in summer. Juvenile eosinophil counts were lower than in adults in winter and spring, and this may be related to lower ecto-parasite loads due to smaller body size of juveniles.

Because of the direct influence of environmental fluctuations, as well as the differing growth and reproductive strategies among males, females and juveniles, it is imperative to assess physiological responses across all seasonal ranges for each cohort. The heterogeneity in survival strategies observed within taxa further highlights the need for species-specific research. This is the first study of this nature on *P. geometricus*, and in addition, the use of digital imaging analyses provided meaningful morphometric measurements, which can contribute towards the standardisation of haematological practice and can be applied in future studies. Baseline haematological values obtained in this study suggest a clinically healthy wild population of geometric tortoises and illuminate the physiological processes of each cohort over all seasons. These results serve as reference data against which abnormal changes can be measured, while an understanding of physiological

responses to environmental change is imperative in the effective management of this Critically Endangered species (Baard 1993). This becomes even more critical in the anticipation of altered weather patterns resulting from global climate change.

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