3 ERYTHROCYTE MEASUREMENTS – SIZE AND SHAPE

3.1 INTRODUCTION

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

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

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

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

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

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

3.2 MATERIALS AND METHODS

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

3.2.1 Histological evaluation of mature erythrocytes

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

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

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

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

3.2.2 Data and statistical analysis

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

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

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

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

3.3 RESULTS

3.3.1 Erythrocyte cell and nucleus size

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

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

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

Parameter	Female	Male	Juvenile
Cell area	135.0, 120.9, 151.2	142.3, 126.6, 159.9	136.4, 122.2, 154.8
Cell length	16.6, 15.5, 17.6	17.0, 15.9, 18.1	16.2, 15.1, 17,3
Cell width	8.2, 7.5, 9.0	8.4, 7.7, 9.1	8.5, 7.8, 9.3
Nuclear area	21.0, 18.2, 24.0	20.9, 18.2, 24.2	22.2, 18.8, 25.9
Nuclear length	5.7, 5.3, 6.1	5.8, 5.4, 6.3	5.9, 5.4, 6.3
Nuclear width	3.7, 3.4, 3.9	3.6, 3.4, 3.9	3.8, 3.4, 4.2

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

	Spring	Summer	Autumn	Winter
Cell				
A	131.5, 117.4, 146.6	130.3, 117.6, 144.7	144.6, 128.4, 163.1	146.1, 132.1,161.1
L	16.4, 15.3, 17.5	16.2, 15.2, 17.2	17.2, 16.0, 18.4	16.9, 15.9, 17.8
W	8.0, 7.4, 8.8	8.0, 7.4, 8.8	8.5, 7.7, 9.2	8.7, 8.1, 9.4
Nucleus				
A	21.6, 18.6, 24.9	18.9, 16.3, 22.7	20.8, 18.6, 23.6	22.8, 20.2, 25,5
L	5.8, 5.4, 6.3	5.5, 5.0, 4.0	5.8, 5.4, 6.2	5.9, 5.5, 6.3
W	3.7, 3.4, 4.0	3.5, 3.2, 3.8	3.6, 3.4, 3.9	3.8, 3.6, 4.1

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

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

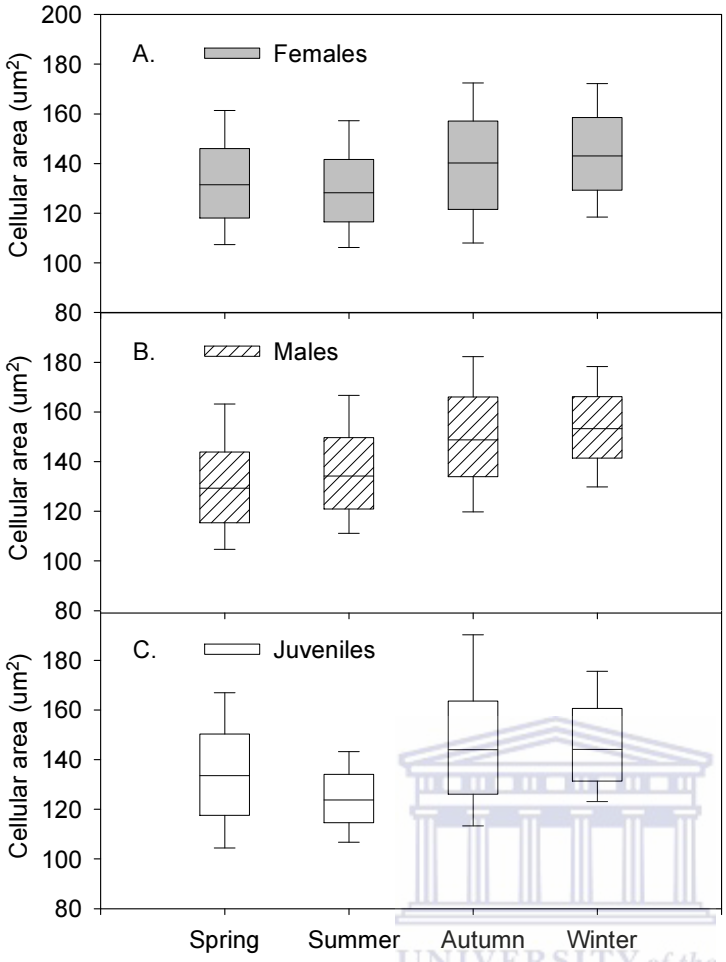

Figure 3.1 Representation of cell area (μm^2) for female (a), male (b), and juvenile (c) *Homopus areolatus* in each of four seasons. The box plot indicates the median, 25% and 75% percentiles and the whiskers represent 10% and 90% percentiles.

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

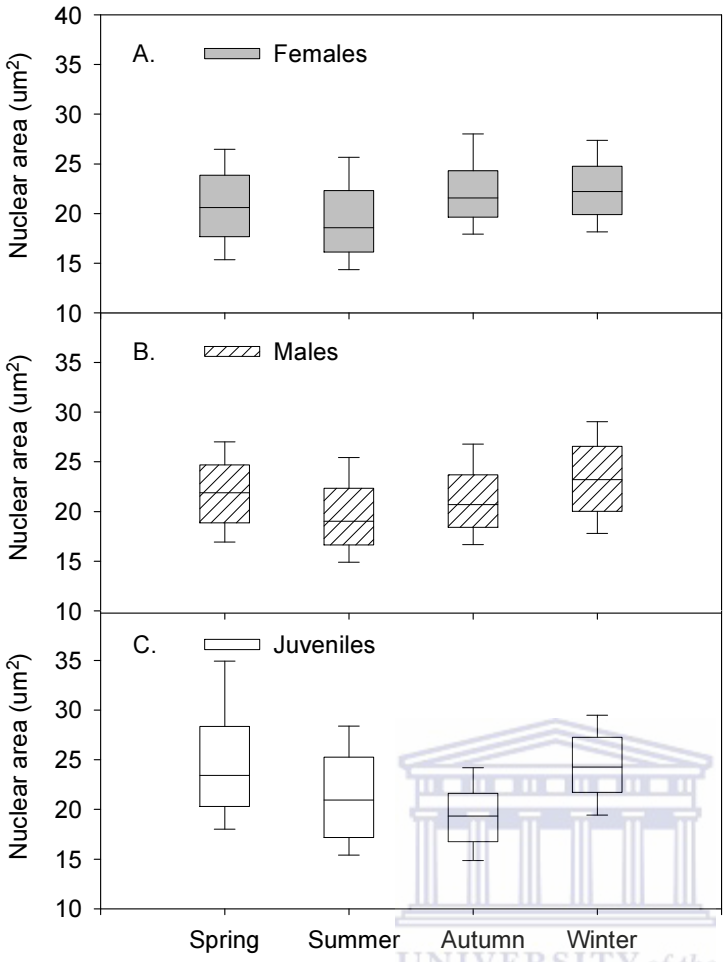

Figure 3.2 Representation of nuclear area (μm^2) for female (a), male (b), and juvenile (c) *Homopus areolatus* within each of four seasons. The box plot indicates the median, 25% and 75% percentiles and the whiskers represent 10% and 90% percentiles.

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

3.3.2 Erythrocyte size classes

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

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

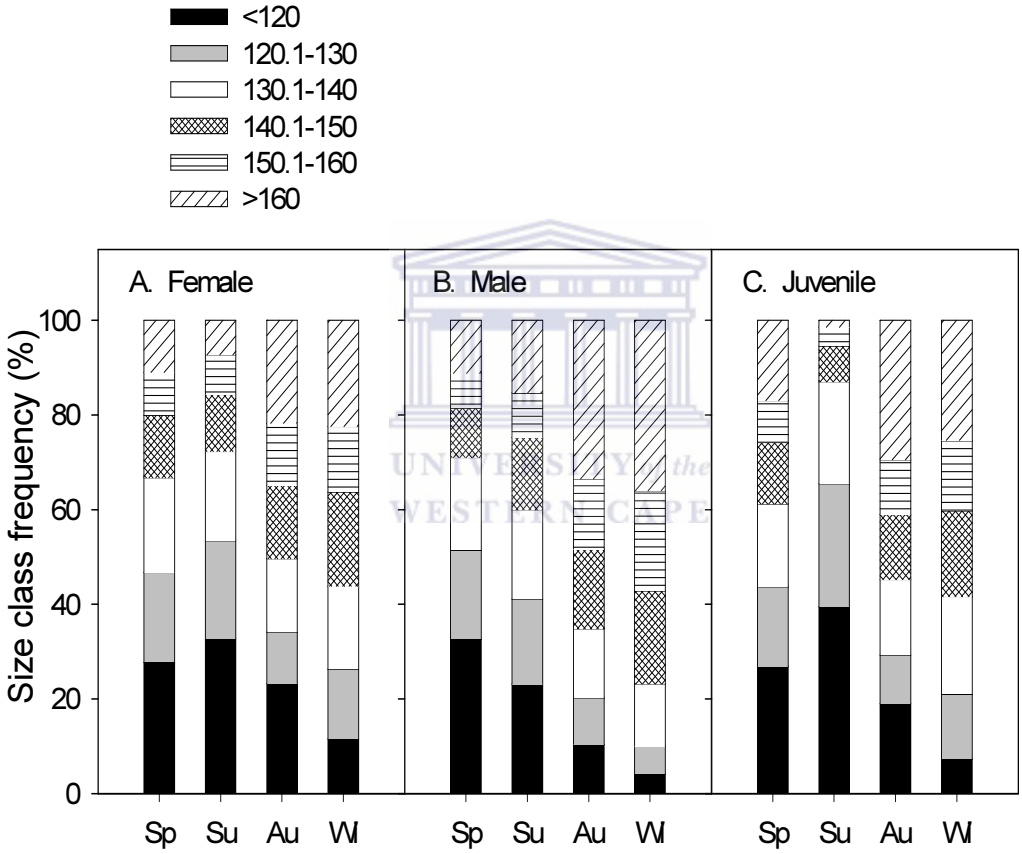

Figure 3.3 Seasonal variation in erythrocyte size classes frequency (%) of *Homopus areolatus* cohorts. Spring is Sp, summer is Su, autumn is Au and winter is Wi.

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

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

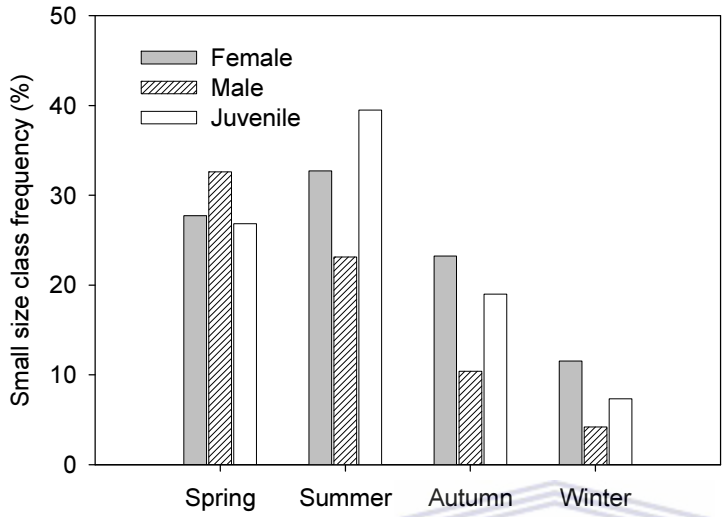

Figure 3.4 Seasonal fluctuations in the frequency (%) of small erythrocytes (<120 um^2) of *Homopus areolatus* females, males and juveniles.

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

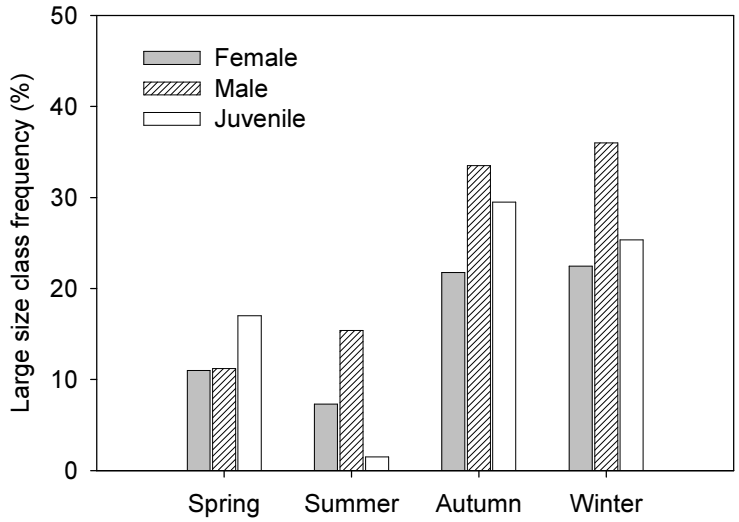

Figure 3.5 Seasonal fluctuations in the frequencies (%) of large erythrocytes (>160 μm^2) of *Homopus areolatus* females (F), males (M) and juveniles (J).

3.3.3 Nuclear to cellular area ratio

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

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

	Spring	Summer	Autumn	Winter
Female	0.16, 0.13, 0.18	0.15, 0.12, 0,17	0.16, 0.13, 0.19	0.16, 0.14, 0.18
Male	0.17, 0.15, 0.19	0.14, 0.12, 0.16	0.14, 0.12, 0.16	0.15, 0.13, 0.17
Juvenile	0.18, 0.15, 0.22	0.17, 0.14, 0.21	0.13, 0.11, 0.15	0.17, 0.15, 0.19

3.3.4 Erythrocyte shape and colour

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

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

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

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

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

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

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

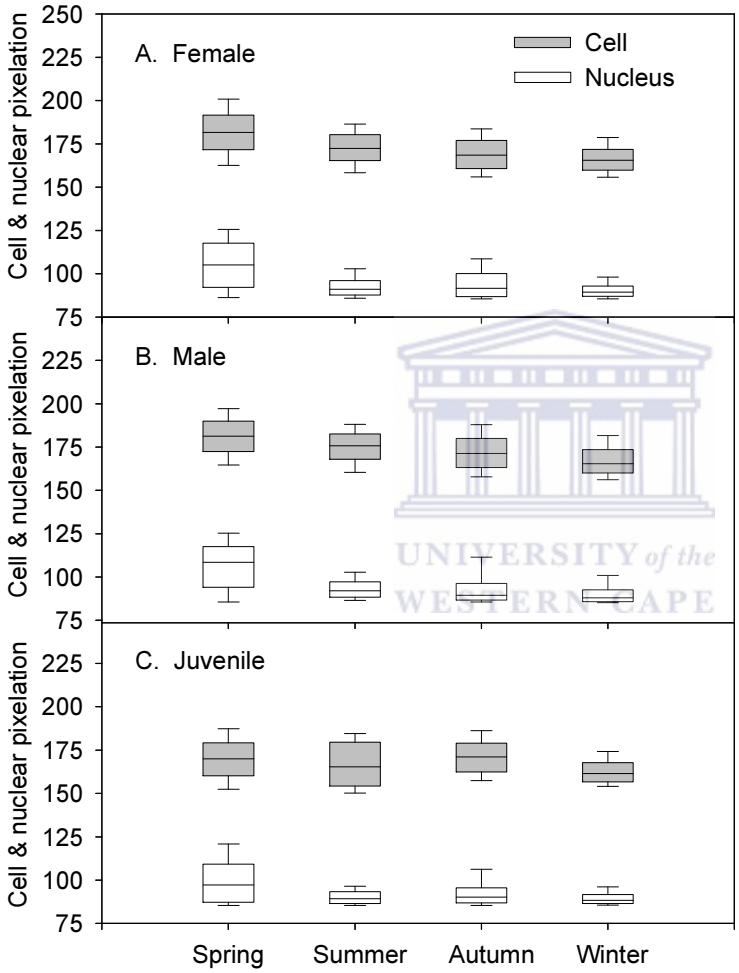

Figure 3.6 Cell and nuclear pixelation for female (a), male (b), and juvenile (c) *Homopus areolatus* over four seasons. The box plot indicates the median, 25% and 75% percentiles and the whiskers represent 10% and 90% percentiles.

3.4 DISCUSSION

3.4.1 Dimensions of mature erythrocytes of *H. areolatus*

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.5 CONCLUSIONS

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

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

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

4 LEUKOCYTE AND THROMBOCYTE HISTOLOGY

4.1 INTRODUCTION

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

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

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

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

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

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

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

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

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

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

4.2 MATERIALS AND METHODS

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

4.2.1 Histological evaluations and differential white cell counts

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

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

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

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

4.2.2 Data and statistical analysis

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

4.3 RESULTS

4.3.1 Leukocyte and thrombocyte morphology

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

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

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

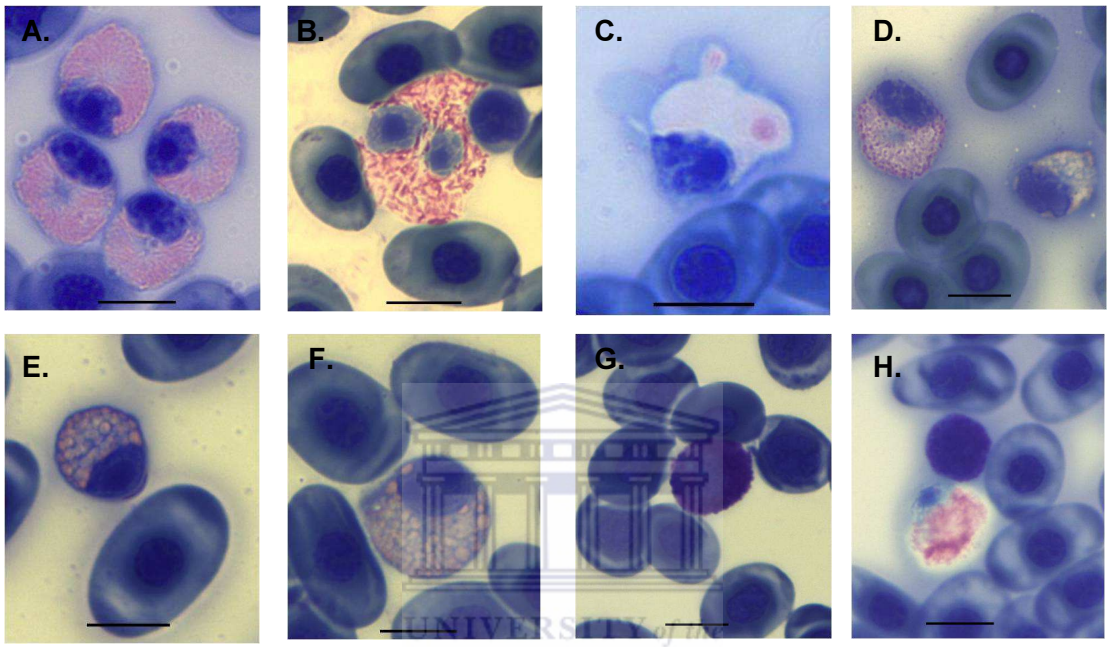

Figure 4.1 Granulocytic leukocytes in the peripheral blood of *Homopus areolatus*. (a) Four uni-lobed heterophils, (b) a bi-lobed heterophil with spindle-shaped granules, (c) a reactive heterophil with a pseudopod and cytoplasmic inclusions and (d) a toxic heterophil on the left with a heterophil on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

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

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

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

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

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

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

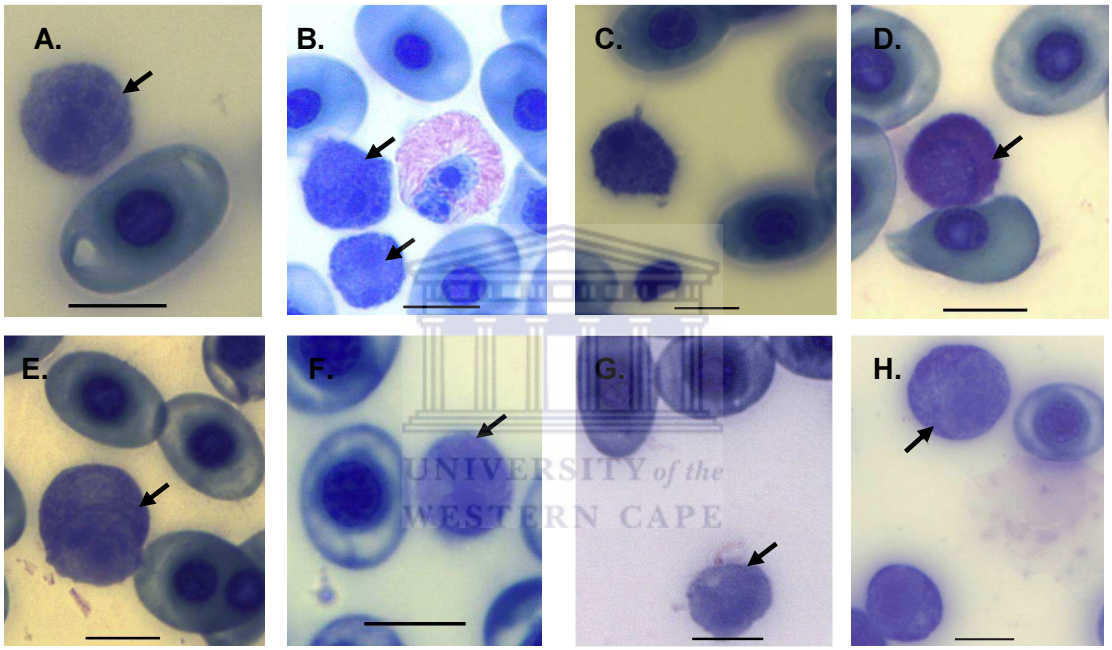

Figure 4.2 Agranulocytic leukocytes in the peripheral blood of *Homopus areolatus.* (a) A lymphocyte showing a large nucleus and thin rim of cytoplasm, (b) two lymphocytes of varying size, (c) a lymphocyte with protrusions, (d,e), plasma cell (f,g) monocytes with typical indented nucleus and basophilic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

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

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

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

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

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

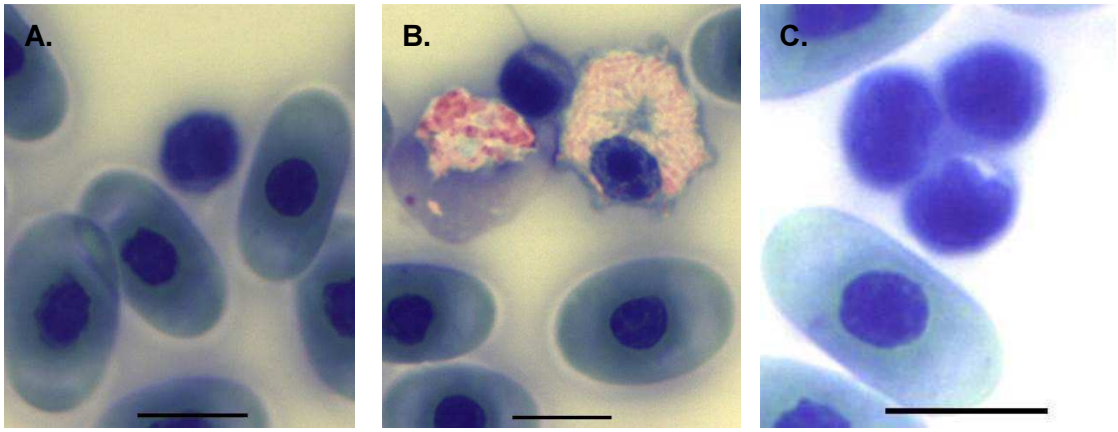

Figure 4.3 Thrombocytes in the peripheral blood of *Homopus areolatus*. (a) A thrombocyte showing a round, large basophilic nucleus with scant rim of clear cytoplasm, (b) thrombocyte with cytoplasmic protrusions, and (c) three thrombocytes in aggregation. The scale bar represents 10 µm at 1000 x magnification.

4.3.2 Cell and nuclear dimensions of leukocytes and thrombocytes

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

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

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

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

4.3.3 Differential white cell counts and effects of season and cohort

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

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

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

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

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

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

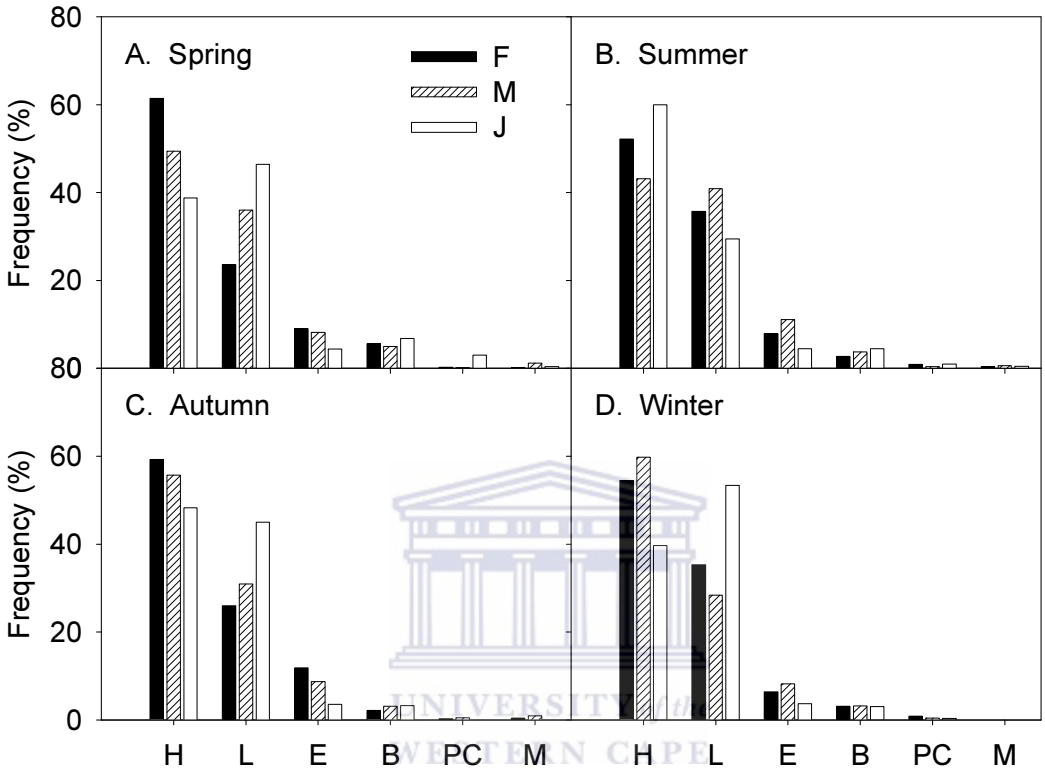

Figure 4.4 Leukocyte prevalence within *Homopus areolatus* among cohorts (female, male, juvenile) and all seasons (spring, summer, autumn, winter). Data presented as percentages.

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

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

4.3.4 Thrombocyte abundance and effects of season and cohort

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

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

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

4.4 DISCUSSION

4.4.1 Leukocyte prevalence and histology

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

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

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

4.4.2 Effects of cohort and season on leukocytes

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

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

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

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

4.4.3 Thrombocyte histology and frequencies

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

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

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

4.5 Conclusions

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

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

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

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

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

5 GENERAL CONCLUSIONS

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

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

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

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

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

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