Influence of blood storage time and temperature on the evaluation of blood smears from Hermann's tortoises *(Testudo hermanni)*

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Für meine Familie

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Abbreviations

A.	*Agrionemys*
ALAT	Alanine aminotransferase
ALP	Alkaline phosphatase
AST	Aspartate aminotransferase
ASVCP	American Society for Veterinary Clinical Pathology
Bact. growth	Bacterial growth
Bas.	Basophils
BCS	Body condition score
BUN	Blood urea nitrogen
Ca	Calcium
°C	Centigrade
Ch.	Chapter
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CK	Creatine kinase
C. v. in ery.	Cytoplasmic vacuoles in erythrocytes
EDTA	Ethylenediaminetetraacetic acid
Eos.	Eosinophils
ff.	First and the following
Fig.	Figure(s)
h	Hour(s)
Het.	Heterophils
ID	Identification
IU	International unit
K	Potassium

kg	Kilogramme
l	Litre
LDH	Lactate dehydrogenase
LMU	Ludwig-Maximilians-Universität
Lymp.	Lymphocytes
μl	Microlitre
μm	Micrometre
Mac. qual.	Macroscopic quality
Mic. qual.	Microscopic quality
m^2	Square metre
ml	Millilitre
Mono.	Monocytes
Na	Sodium
P	Phosphate
PAS	Periodic acid Schiff
PCR	Polymerase chain reaction
Stor. temp.	Storage temperature
Stor. time	Storage time
syn.	Synonymous
T.	*Testudo*
Thromb.	Thrombocytes
TP	Total protein
U	Unit(s)
UA	Uric acid
UV-B	Ultraviolet B

1. Introduction and aims

Reptile husbandry in Germany has been on the rise for several years now, with tortoises representing one of the most popular species group. These animals are more and more recognised as pets and equivalently cared for, with owners more willing to undertake any efforts for the well-being of their pet tortoise. This leads to tortoises being presented more frequently at veterinary surgeries. Within the tortoises, the Hermann's tortoise, *(Testudo hermanni)* is one of the most widespread species in captive husbandry. This also becomes evident in veterinary surgery, where Hermann's tortoises represent a large part of the chelonian patients. At the Klinik für Vögel, Reptilien, Amphibien und Zierfische of the LMU Munich, 456 chelonians were presented within one year (July 2014-June 2015) and 275 of these were Hermann's tortoises (60,3 %). For an ethically and biologically correct captive husbandry of these exotic animals, the access to a specialised, scientifically well-grounded medical care is essential to maintain the health of the animals. Providing this medical care seems all the more important with regard to the current debate about the captive husbandry of reptiles in private ownership and the consequential possible initiation of positive or negative lists in Germany. Given that reptile medicine covers an extremely large range of species and therefore species-specific health issues, fundamental research such as the presented study is still necessary.

During the clinical assessment of reptilian patients, the haematologic examination is of great importance, since reptiles as wild animals only start showing signs of illness in advanced stages of disease. When reviewing a tortoise patient, the possibilities of continuative examinations are limited due to the shell, so that a blood examination is essential to permit an estimation of the tortoise's health status. In the context of a blood

evaluation, the blood smear is given priority [Stacy et al., 2011], because the microscopic examination of a blood smear can yield many results that are clinically relevant.

Reptiles as ectothermic animals still pose a challenge to the practitioner when it comes to blood evaluations. In comparison to mammals, the metabolic processes in ectothermic animals are influenced far more by environmental conditions, which might be reflected in changes of blood parameters. In addition, to obtain reliable and useful information from a blood smear, the handling of the samples is critical, since artefacts from handling may also influence the obtained results. Thus, it is difficult to determine whether a deviating blood value results from an external influence or is actually a pathological sign. The large amount of reptile species in differing habitats with different lifestyles provides for large ranges of blood values and makes it impossible to deduce from one species to another.

While several investigations have recently been performed to determine the influence of sex, reproductive status or season on blood parameters in reptiles (for example [Lawrence and Hawkey, 1986; Lawrence, 1987; Anderson et al., 1997; Christopher et al., 1999; Chung et al., 2009; Scope et al., 2013]), the impact of external factors acting on blood after sampling are less known. This study was performed to determine the influence of the storage time and storage temperature on the interpretability and results in blood smears from *T. hermanni*, with emphasis on the differential blood count and the morphological changes in the blood cells. Also, a photographic documentation of the blood cells of *T. hermanni* was pursued, including the changes resulting from prolonged storage at fridge and room temperature.

2. Literature

2.1. Hermann's tortoises

Hermann's tortoises *(Testudo hermanni)* are native to mediterranean Europe. The distribution area ranges from northeast Spain and southern France across Italy, Corsica, Sardinia, Sicily and the larger part of the Balkan peninsula to the west coast of the Black Sea. The habitats are variable and range from dry, stony areas to more densely overgrown and partly forested areas. Floodplains and other wetlands as well as areas characterised by human utilization such as gardens, fields, olive groves and vineyards are also frequently inhabited. The climate is mostly mediterranean with dry, hot summers and mild winters. An exception to this are the higher mountain regions in the Balkans. The nutrition consists mainly of a wide range of herbaceous plants, but on occasion invertebrates such as snails and worms are also ingested as well as rotten carcasses. The tortoises hibernate at least from November till February, although in the warmer southern areas some individuals may also be seen during sunny and mild winter days [Glandt, 2009].

T. hermanni is listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), Appendix II, as a species that is not directly in danger of extinction but this may change unless the international trade is closely controlled [CITES, 2015]. For Germany, this means that a certificate of permission is required by the correspondent authorities for an import of these animals as well as export documents from the state of origin. Each animal must have its own proof of origin [Bundesamt für Naturschutz, 2015].

2.2. Blood sampling and evaluation

In reptiles, the total blood volume is about 5-8 % of the body weight [Heard et al., 2004]. Recommendations for a maximum blood volume that can be collected safely from reptiles vary from 3 ml [McArthur, 2004] or 4 ml [Kölle, 2014] to even 5-8 ml per kg body weight [Heard et al., 2004]. 1-1,5 ml of blood per kg body weight are recommended for daily practice, as this volume is sufficient for elaborate chemistry and haematology evaluations [McArthur, 2004].

2.2.1. Venipuncture sites in tortoises

Blood sampling in tortoises may appear difficult due to the bony carapace and the scarce possibilities to visualize peripheral blood vessels. Over time, researchers developed and tested several possibilities to obtain blood samples from tortoises and a few are described here.

Decapitation, generally irrelevant for veterinary practice nowadays, was used by researchers in the past to collect blood samples [Duguy, 1970; Leceta and Zapata, 1986]. Blood sampling from clipped nails, toes or tails should not be performed with regards to animal welfare and ethical aspects. Nail-clipping was nevertheless regarded as the only possibility available in very small reptiles with a body weight below 30 g [Campbell and Ellis, 2007]. It is not entirely clear if the blood results are impaired when using blood samples from short-clipping a nail. No differences were found in *Testudo graeca* and *T. hermanni* when comparing samples from this method with samples obtained from the Vena coccygealis dorsalis [Lawrence and Hawkey, 1986]. However, according to Campbell and Ellis [2007] this site does not provide optimal samples for haematologic evaluation.

For the clinician, the jugular vein was recommended as the first choice for tortoises, since lymphodilution is least likely in this site [McArthur, 2004]. It is also the only vein that is generally visible or can be visualised in tortoises [Jacobson et al., 1992]. To access the jugular vein, the head and neck need to be drawn out and restrained securely behind the

head. The vein extends caudally from the mandibular angle, roughly in a line parallel to the neck from the dorsal border of the tympanic membrane to the spot where neck and carapace meet. The needle is inserted superficially in a caudal direction. Overly compression of the vein is not neccessary and may even lead to large haematoma when the needle is taken out. In uncooperative chelonians, jugular venipuncture is inapplicable without chemical restraint [McArthur, 2004].

The forelimbs may be easier to access than the head in uncooperative tortoises, giving the option to draw blood from the brachial vein. In this site the risk of lymphodilution is high and sometimes pure lymph may be obtained [Jacobson et al., 1992]. The brachial venous plexus is located at the flexor aspect of the elbow. The needle is inserted into the V-shaped junction of the biceps tendon and the radius. In smaller tortoises, an acute angle must be maintained, while a perpendicular angle is required in large tortoises [Heard et al., 2004].

Blood sampling from the dorsal coccygeal vein does present a considerable risk of lymphodilution, but it is often the only site available without chemical restraint and can be accessed blind in the dorsal median line of the tail, when the tortoise absolutely refuses to present its tail. In this site, disinfection of the venipuncture site prior to the sampling was regarded as especially important, as the blood vessels in the tail are at least partially drained by the renal portal vein. It was suspected that renal infections may follow blood sampling from the dorsal coccygeal vein when pathogens inoculated during venipuncture are transported to the kidneys [McArthur, 2004]. To access the dorsal coccygeal vein, the needle is inserted in the dorsal median line of the tail. Gentle suction is maintained and when the vein is located, the blood normally flows freely [McArthur, 2004].

The Sinus venosus subcarapaxialis has been identified by Hernandez-Divers et al. [2002] and is considered a very useful blood sampling site, especially when other sites have proven difficult to access. The risk of lymphodilution is not fully clarified yet in this sampling site, but there are lymphatic vessels running directly cranial to the sampling area [McArthur, 2004]. This blood collection site also holds a certain risk of haemorrhage from the lungs, if these are punctured accidentally. The needle is inserted in the median, directly below the transition between skin and carapace, following a caudodorsal direction.

A negative pressure is maintained. Redirection of the needle is neccessary when touching the underside of the carapace or a vertebra [Hernandez-Divers, 2006]. The possible complication of pulmonary haemorrhage when misdirecting the needle should not be disregarded in this sampling site.

Blood sampling by cardiocentesis does not have a high relevance for veterinary practice because the risks of contamination with following pericarditis and death of the animal need to be considered carefully and a sterile approach is neccessary. Under anaesthesia, a hole is drilled into the plastron with a sterile drill. After the sampling, the hole must be sealed with bone wax and a methacrylate resin or other suitable sealants [Jacobson et al., 1992]. In juvenile tortoises with soft plastrons, complications are unlikely when puncturing the plastron with a needle, but other sampling sites such as the subcarapacial sinus should be preferred in these cases. Small hatchlings or neonates did not indicate pain and/or distress during cardiocentesis, whereas large tortoises are known to fidget wildly when simply touching the plastron with a needle. Analgesia must be considered in such cases, if other sampling sites are no option [McArthur, 2004].

2.2.2. Influence of the handling of blood specimens on results

The American Society for Veterinary Clinical Pathology (ASVCP) has published Quality Assurance Guidelines on how to handle blood samples generally [ASVCP, 2009]. Information on proper handling of reptilian blood is very scarce. It is just noted that samples should be sent in a timely manner to the evaluating laboratory, with storage conditions suitable for the particular sample type. For avian blood samples, the reasonable transport time is indicated to be shorter than the transport time for mammalian and reptilian blood samples. The ASVCP Quality Assurance Guidelines refer to a controlled study, where the influence of three different anticoagulants (sodium citrate, EDTA, lithium heparin) on the storage of blood samples from macaws and burmese pythons *(Python molurus bivittatus)* was compared. The avian blood samples showed a strong haemolysis within twelve hours of storage, independent of anticoagulant. The blood samples from burmese pythons collected in EDTA or heparin were more stable up to 24 hours [Harr et al.,

2005]. According to this data obtained from blood storage of a single snake species, a generalised storage duration of up to 24 hours was recommended by the ASVCP, before reptilian blood samples start to deteriorate [Vap et al., 2012].

While automated counting techniques are widely used in mammalian medicine, blood samples of reptiles as well as birds present the problem of nucleated red blood cells. The automated counting techniques are based on separating the nucleated white blood cells from non-nucleated red blood cells and therefore do not work with nucleated red blood cells. On account of this, manual methods are still used for the most part. The quality and correct interpretation of results obtained from blood smears are strongly dependent on preparation of blood smear, staining methods and absence of artifacts [Houwen, 2000; Murray, 2000]. The used methods for blood sampling and specimen handling should be consistent as well as the choice of the laboratory for the chemical analysis [Murray, 2000].

The choice of blood smear technique has been found to influence the quality of the blood smear. When three different smear techniques were compared in blood smears from green iguanas *(Iguana iguana)*, a coverslip-to-slide technique and a bevel-edge slide technique provided the best results regarding monolayer quality and smudge cell counts. A slide-to-slide technique however, yielded higher smudge cell counts and lower monolayer quality than the other two techniques and was therefore not recommended [Perpiñán et al., 2006].

In another study six different smear techniques in chicken blood samples were compared. Significantly lower smudge cell counts were found when using a wedge-smear technique with two slides at an angle of 45° compared to the same technique with a coverslip instead of a slide. The comparison with a coverslip-to-slide technique where the blood drop was placed in the first third of the slide also showed significantly lower smudge cell counts. The wedge-smear technique with an angle of 30° and 45° each showed a distinctly higher amount of squashed cells when the top slide was pushed before the blood had fully spread across the bottom slide instead of waiting until the blood drop had fully spread across the slide edge, which yielded better results [Reauz et al., 1999].

The aforementioned study also found the method of cell counting to influence the result in combination with the smear technique and the examined area of the blood smear. A coverslip-to-slide technique with the blood drop placed in the first third of the slide showed a significant change in heterophil, lymphocyte and monocyte values when comparing the count of 100 and 400 leucocytes in the optimal area of the blood smear. This increase of the differentiated leucocytes from 100 to 400 did not influence the accuracy of the counting results in the wedge-smear techniques when using the optimal area. An additional conclusion from this study was that not only the smear method and the quality of the smear are of significance to the results, but also the examined area of the smear. The middle vertical third of the blood smear was the optimal area in the wedge-smear technique with the top slide pushed at an angle of 45° after complete spreading of the blood drop along the edge of the top slide. This area was also best in the coverslip-to slide technique with the blood drop placed in the first third of the slide. The optimal area for a wedge-smear technique using a coverslip instead of the top slide was the lower horizontal third of the blood smear [Reauz et al., 1999].

Venipuncture site has also been found to influence haematologic and biochemical blood parameters. In marginated tortoises *(T. marginata)* a significant difference in red blood cell count, packed cell volume and haemoglobin values was determined between the dorsal coccygeal vein and the brachial vein as blood collection sites. The biochemical values total protein, uric acid, calcium, phosporus, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, alkaline phosphatase, calcium and phosporus were increased in the brachial vein samples compared to the samples from the Vena coccygealis dorsalis. In this study, haemodilution due to lymph was assumed to be the reason for the differing values. Therefore it was concluded that in marginated tortoises the brachial vein is more suitable than the dorsal coccygeal vein to obtain reliable blood results [López-Olvera et al., 2003].

Further factors not directly related to the animals have also been proven to influence blood parameters. When the effect of anticoagulant was evaluated in loggerhead sea turtles *(Caretta caretta)*, a significant difference was observed in the glucose values obtained from lithium-heparinised samples and from sodium-heparinised samples [Bolten et al., 1992].

Significant differences were reported from the red blood cell counts in Hermann's tortoises *(T. hermanni)* when comparing samples stored in EDTA and heparin. Here, the red blood cell counts were lower in the EDTA samples due to the haemolysis associated with EDTA in reptilian blood samples. Differential leucocyte counts were obtained only from the heparin samples, since these provided optimal blood smears while those from the EDTA samples were not evaluable due to the haemolysis [Muro et al., 1998].

Although there are not many reports about the influence of storage condition on blood samples, especially in reptiles, in one study the effects of storage on the sodium and potassium values in Aldabra tortoises *(Geochelone gigantea)* and Burmese mountain tortoises *(Manouria emys)* were investigated. The authors stored blood samples at 4 °C and at 25 °C for 5, 15, 30, 60 and 120 minutes after collection of the blood sample. Half of these blood samples were stored in lithium heparin and the other half without an anticoagulant. When investigating the sodium and potassium concentrations, no differences were found for potassium concentrations in Aldabra tortoises for all storage conditions. The sodium concentrations declined significantly in samples with no anticoagulant stored at 4 °C and 25 °C, as well as in heparinised samples stored at 4 °C. In the Burmese mountain tortoises the potassium concentrations were increased with time in both storage temperatures, but the increase was lower in 4 °C. The sodium concentrations decreased significantly with time in all storage conditions [Abou-Madi and Jacobson, 2003].

Influence of storage temperature on haematocrit, haemoglobin concentration and red blood cell count as well as total white blood cell count has been documented for blood samples from cattle, goats and pigs stored at 30 °C and 5 °C. Significant changes were described in the haematocrit after 14 hours of storage at 30 °C and after 19 h at 5 °C in bovine and caprine blood samples. The blood samples from pigs showed a significant change in the haematocrit after 10 h of storage at 30 °C and 14 h at 5 °C. Significant changes in the haemoglobin concentration were found when storing porcine samples for 96 h at both temperatures. The porcine samples also showed significant changes in the red blood cell count after 48 h at 30 °C and 96 h at 5 °C. The total white blood cell count showed signifant changes in blood samples from goats after storage for 120 h at 30 °C and 5 °C. In any case, the artifactual changes were more distinct in those samples with a storage temperature of 30 °C [Ihedioha and Onwubuche, 2007].

After storing canine blood samples for up to 48 h at room temperature, no significant changes could be found for the red blood cell count and the haemoglobin concentration. The mean corpuscular volume increased throughout the study in almost all specimens. The white blood cell counts remained unchanged or were higher in two thirds of the samples after 24 and 48 h and were lowered in the remaining third of the samples. The platelet counts were found to be significantly decreased over the first 24 h and afterwards remaining stable for the next 24 h [Médaille et al., 2006].

For human patients with thrombocytopenia it has been reported that the thrombocyte count was lowered further in vitro due to storage at room temperature for 90 minutes. The platelet counts were decreased after storage at room temperature for 90 minutes in both anticoagulants evaluated (EDTA and citrate-tris-pyridoxalphosphate). The samples stored in EDTA showed a higher increase in platelet agglutination compared to those stored in citrate-tris-pyridoxalphosphate. This phenomenon was of clinical relevance and lead to problems in the interpretation of the blood values and the following choice of treatment [Podda et al., 2012].

In another study human blood samples were stored for 0, 4, 24 and 48 h at 4 °C, room temperature and at 35 °C, before separating the cellular fraction from plasma and serum and measuring inflammatory markers. This procedure showed a significant increase in the concentration of several cytokines when samples were stored for a period of time before centrifugation. Plasma concentrations of some cytokines were increased already after 4 h storage at 4 °C and more cytokines were increased higher when the samples were stored at room temperature. For the storage at 35 °C, all cytokines were increased compared to the storage at 4 °C, but decreased compared to the storage at room temperature. The serum concentrations showed a generally higher increase in the analytes compared to plasma. All in all, the serum concentrations were least increased after the storage at 4 °C and most increased after the storage at 35 °C [Skogstrand et al., 2008].

2.2.3. Further factors influencing blood parameters in reptiles

Many previous studies revealed a great range of variability in reptilian blood parameters due to very different influencing factors, for example season, species, reproduction status, sex and blood sampling site.

Seasonal variation of haematologic parameters were indicated in a study on long-term captive spur-thighed tortoises *(T. graeca)* and Hermann's tortoises *(T. hermanni)* in the United Kingdom. The red blood cell count, packed cell volume, and haemoglobin values decreased during spring to early summer, then remained at a low level till October and increased in November. In December an abrupt decline was found with a concurrent rising of the mean corpuscular volume. The mean corpuscular haemoglobin concentration sank during hibernation and rose again in the summer. The total white blood cell count rose in autumn and declined during hibernation before it rose again in summer. Heterophil and lymphocyte counts were decreased between January and March and during the active period in summer the lymphocytes were increased. In October there was also a rise of eosinophils [Lawrence and Hawkey, 1986].

In gravid female spur-thighed tortoises *(T. graeca)* and Hermann's tortoises *(T. hermanni)* a significant increase in total protein, plasma lipids and cholesterol was determined around the time of egg deposition in August. Significant changes in urea and glucose were found in context with hibernation, the values were significantly increased in April during awakening. The urea levels had already begun to rise in March, while the glucose levels were more closely associated with awakening [Lawrence, 1987].

Significant species differences in chemical blood parameters were reported for Hermann's tortoises *(T. hermanni)*, spur-thighed tortoises *(T. graeca)*, marginated tortoises *(T. marginata)* and russian tortoises *(T. horsfieldii)* in 10 out of 20 tested parameters. Uric acid, urea, total bilirubin, cholinesterase and sodium showed highly significant differences, while the values for creatine kinase, fructosamine, glucose and potassium showed high significant differences. Alkaline phosphatase was reported to show a significant difference between the four species [Mathes et al., 2006].

Another study revealed relevant differences in *T. hermanni* between male and female individuals. Triglycerides, cholesterol, calcium and inorganic phosphorus were found to be significantly higher in females over two summer seasons. Aspartate aminotransferase, alanine aminotransferase, bile acids, lactate dehydrogenase and glutamate dehydrogenase were increased in male tortoises during the mid-summer months. In both sexes, however, alanine aminotransferase, uric acid and urea concentrations varied significantly during the seasonal cycles [Scope et al., 2013].

In clinically normal, captive New Guinea snapping turtles *(Elseya novaeguineae)* that were kept at different temperatures, a significant difference in certain blood parameters was reported. Creatine kinase, albumin, potassium and phosporus values obtained from the animals kept at 24.5 °C proved to be higher than from the animals kept at 30 °C. Cholesterol and calcium values were reported to be higher in females than in males while haemoglobin, packed cell volume and bilirubin values were higher in males than in females. Furthermore, the bile acid values were found to be higher when evaluating serum samples rather than plasma samples [Anderson et al., 1997].

In free-ranging desert tortoises *(Gopherus agassizii)*, significant differences between males and females were reported for packed cell volume, haemoglobin concentration, aspartate transaminase activity and cholesterol, triglyceride, calcium and phosphorus values. The reproductive cycle, hibernation and seasonal rainfall also provoked a marked variation in most blood parameters tested [Christopher et al., 1999].

The Asian yellow pond turtle *(Ocadia sinensis)* showed sex-specific differences in packed cell volume, eosinophil count, percentages of heterophils and monocytes as well as total protein, albumin, uric acid, aspartate aminotransferase, alanine aminotransferase, triglycerides, cholesterol and alkaline phosphatase. A strong seasonal variation was reported as well for most chemical blood parameters [Chung et al., 2009].

2.2.4. Considerations during interpretation of reptilian blood values

Many aspects need to be given careful consideration when evaluating reptile specimens. The great number of recent reptile species, 10,727 as of August 2015 [Uetz and Hošek, 2015], already hints toward the impossibility of establishing valid reference values for every species. Exemplary reference values for the differential blood count from several literature sources are provided in Table 2.1. External factors, such as environmental conditions, need to be considered as well as internal factors, such as age and gender. Several examples were described already in the previous section.

In comparison to homoiotherm or endothermic animals, reptiles as exothermic animals have less stable cellular microenvironments which makes their cellular responses to disease far more unpredictable. The variability of reptilian blood values is further enhanced by the influence of sample-handling factors, such as choice of anticoagulant, type of stain, blood collection site and cell counting method. All these influence factors increase the difficulty in establishing reference ranges for reptiles, that can be generally accounted for as normal blood values. When evaluating haematologic results in a reptile patient, total and differential leucocyte counts were therefore said to have to show twofold or greater deviation from reference values to be regarded as significant [Campbell, 2006].

Nevertheless, without existence of reference ranges, comparative haematology is most useful in assessing the progress of a reptile patient in disease and the reaction to therapy by comparing the course of parameters in repeated samplings. An alteration in the total leucocyte count from leucocytosis or leucopenia towards normal values is always an eligible reaction. Likewise, normal heterophil or eosinophil counts after heterophilia or eosinophilia are a sign of an improvement in the health status of the patient. A positive reaction to treatment would also be the disappearance of toxic heterophils and reactive lymphocytes in the blood smear. Consequently, haematologic evaluation can serve as an useful indicator in estimating the progress of a reptile patient [Campbell, 2006].

Table 2.1. Leucocyte reference ranges of some tortoise species from several literature sources (Differential blood counts given as percentages).

Species	Heterophils	Eosinophils	Basophils	Lymphocytes	Monocytes	Author
reptiles in general	15-60	3-50	0-12	18-50	0-8	Kölle [2014] (recommended ranges)
Testudo hermanni	winter: 42-83 spring: 12-83 summer: 34-80	winter: 0-2 spring: 0-30 summer: 0-5	-	winter: 15-58 spring: 12-50 summer: 20-64	winter: 0-2 spring: 0-10 summer: 0-2	Heard et al. [2004]
Testudo hermanni and *Testudo graeca*	January: 42-83 March: 12-83 June: 34-80 October: 16-56	January: 0-2 March: 0-30 June: 0-5 October: 0-50	-	January: 15-58 March: 12-50 June: 20-64 October: 29-52	January: 0-2 March: 0-10 June: 0-2 October: 0-4	Lawrence and Hawkey [1986]
Testudo hermanni	-	17-23	8-15	42-50	20-28	Tosunoğlu et al. [2005]
Testudo graeca	-	11-28	5-20	35-44	20-28	Tosunoğlu et al. [2005]
Gopherus agassizii	15-57	0-4	4-28	17-43	4-20	Jacobson et al. [1991]

2.3. Reptilian haematology

Below, the cell types of reptiles are described and the descriptions are summarised at the end (Table 2.2). Additionally, a microscopic photo is provided as an example for the morphological differentiation of blood cells in *T. hermanni* (Fig. 2.1).

2.3.1. Erythrocytes

Reptilian erythrocytes in general are oval cells with rounded poles and homogenous translucent cytoplasm. The nuclei show a rounded, more or less irregular shape and are located centrally in the cell [Saint Girons, 1970]. The colour of the cytoplasm with the Wright's stain in *Agrionemys horsfieldii* (syn. *Testudo horsfieldii*, in the presented study the name *A. horsfieldii* is used) was described as light and dark pink [Shadkhast et al., 2010] or light yellowish pink in *T. graeca* and *T. hermanni* [Arikan et al., 2015]. In Pappenheim-stained smears the cytoplasm was described as orange-pink in *A. horsfieldii* [Knotková et al., 2002]. The nuclei were described as dark purple in both stains. Erythrocyte life span in reptiles is much higher than in mammals (2-5 months) and birds and can reach up to three years [Frye, 1991].

The size of reptilian erythrocytes in general ranges from a length of 14-23 μm and a width of 8-14 μm [Saint Girons, 1970; Campbell, 2006]. In *T. hermanni* the size of the erythrocytes was indicated with a mean length of 19.2 μm and a mean width of 10.9 μm [Arikan et al., 2015]. In *T. graeca* the size has been measured with a length of 18.5 μm and a width of 10.6 μm [Saint Girons, 1970]. In *A. horsfieldii* the size was indicated with 19.5 μm $\pm$ 1.2 μm length and 9.2 μm $\pm$ 1.0 μm width [Knotková et al., 2002].

2.3.2. Thrombocytes

Thrombocytes are small, oval cells with central, highly chromophilic nuclei and scant amounts of cytoplasm. The cytoplasm was described as lightly acidophilic, almost colourless, and frequently showing azurophilic granules [Saint Girons, 1970]. Other authors characterised the cytoplasm as faintly basophilic and the cell shape as more angular than in lymphocytes, with one or more projections [Alleman et al., 1992]. Knotková et al. [2002] wrote about two forms of thrombocytes found in *A. horsfieldii*, one with the cytoplasm loosely curled around the nucleus and the other with elliptical cytoplasm with a clearly visible membrane. Other researchers described differently shaped thrombocytes in two species: *T. hermanni* showed spherical thrombocytes while *T. graeca* had spindle-shaped thrombocytes [Arikan et al., 2015].

The size of thrombocytes was described with a mean length of 12.0 μm and a mean width of 6.8 μm in *T. hermanni* and a mean length of 12.6 μm and a mean width of 7.4 μm in *T. graeca* [Arikan et al., 2015]. The differentiation of thrombocytes and lymphocytes can be difficult in reptiles, but in most species they stain positively with Periodic acid Schiff (PAS) stain, while lymphocytes stain PAS-negative [Alleman et al., 1992].

2.3.3. Leucocytes

Classification of leucocytes

The classification of leucocytes in reptiles is not always handled explicitly. It is complicated further by the morphologic variation of leucocytes among different reptilian species [Campbell and Ellis, 2007]. In literature, there are varying descriptions of the nomenclature in leucocytes, especially granulocytes. Some authors specified eosinophilic, basophilic and neutrophilic granulocytes [Saint Girons, 1970; Wood and Ebanks, 1984] while others defined heterophilic, eosinophilic and basophilic granulocytes [Knotková

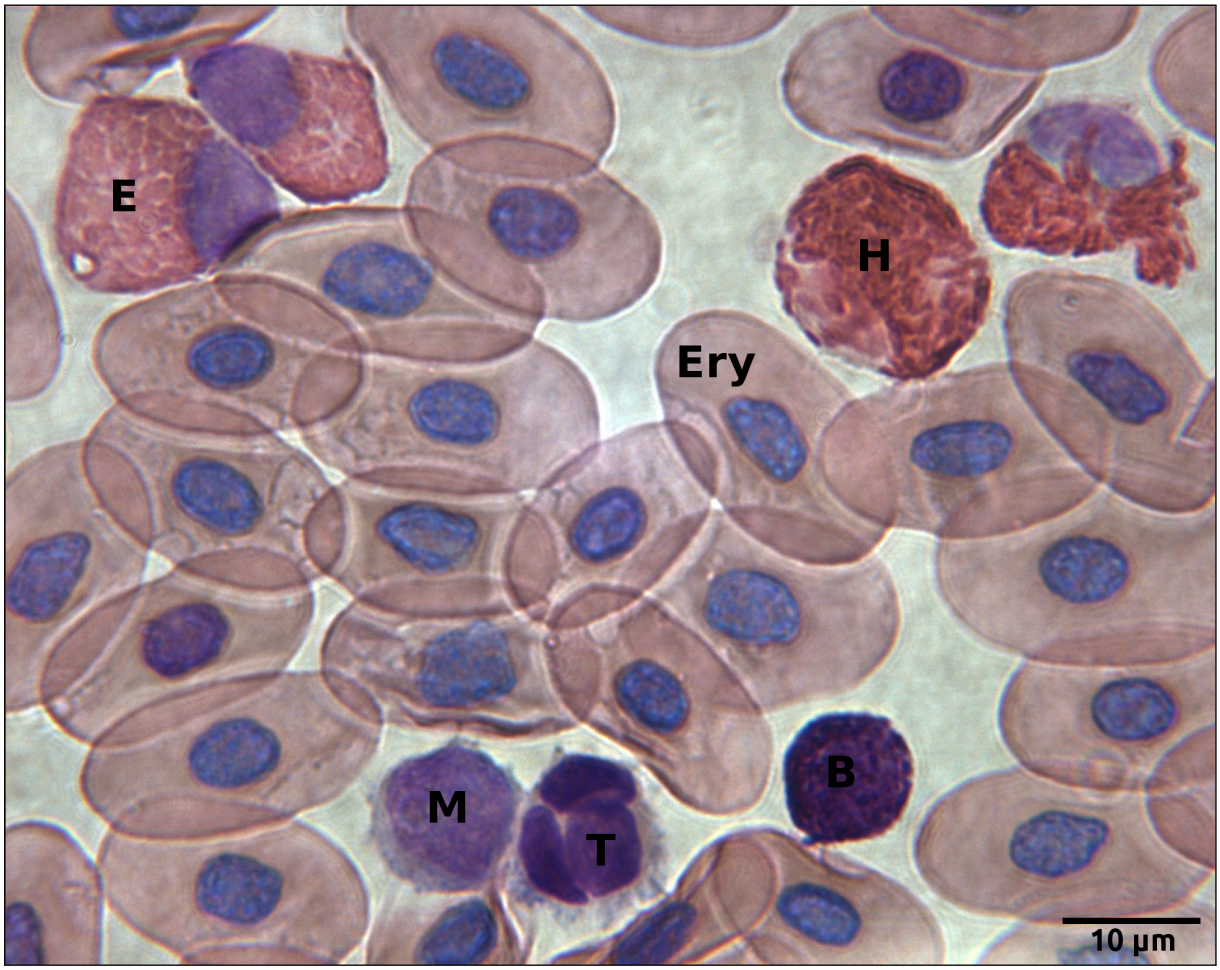

Fig. 2.1. Morphological differentiation of blood cells. E = eosinophil, H = heterophil, M = monocyte, T = thrombocyte, B = basophil, Ery = erythrocyte.

et al., 2002]. Heterophils, eosinophils, basophils and neutrophils as granulocytes that can be differentiated are listed by Kölle [2005].

Saint Girons [1970] described two types of eosinophilic granulocytes and remarked that one of these has also been described as heterophils. This statement might also explain the absence of heterophils in the classification of leucocytes in older texts. The nomenclatorial confusion has been increased further as the neutrophilic granulocytes may also be regarded as a morphological variation of the azurophilic granulocytes and have also been described as heterophils [Ryerson, 1949; Saint Girons, 1970].

Apart from the granulocyte classification there is no conclusive agreement about plasma cells as a separate cell group. Some authors, for example Alleman et al. [1992]; Raskin [2000]; Knotková et al. [2002]; Shadkhast et al. [2010]; Arikan et al. [2015], did not mention plasma cells at all, in some works they were described as belonging to lymphocytes [Heard et al., 2004; Campbell and Ellis, 2007] and other researchers mentioned plasma cells as a usually rare cell type that may arise from medium-sized or large lymphocytes [Saint Girons, 1970].

In this work, the classification as presented in several educational books was adopted that divides the leucocytes first into granulocytes and mononucleated leucocytes [Campbell, 2006; Campbell and Ellis, 2007; Hnízdo et al., 2011]. The granulocytes are then further separated into acidophilic and basophilic granulocytes, where the acidophilic granulocytes are then divided into heterophils and eosinophils. The mononucleated leucocytes are divided into lymphocytes and monocytes [Campbell and Ellis, 2007]. Plasma cells were accounted for by some authors [Saint Girons, 1970; Frye, 1991; Campbell and Ellis, 2007; Nardini et al., 2013; Joseph, 2015] and were also considered.

There is still some confusion about azurophils. Saint Girons [1970] described them as a separate type of granulocyte. Some authors considered these monocytoid cells as specific to iguanas and many snake species [Raskin, 2000], while others listed them as unique to snakes [Heard et al., 2004]. In green iguanas (*Iguana iguana*) different monocytoid cells were described, partly with azurophilic granules, and they have been discussed to be termed as monocytes, including all of the cells within this cell line [Harr et al., 2001]. Nevertheless, there are also reports of few azurophils found in chelonians, for example *A. horsfieldii* [Knotková et al., 2002], *T. hermanni* [Neiffer et al., 2005] and *Elseya novaeguineae* [Anderson et al., 1997]. Azurophils are not further considered in this work.

Heterophils

Heterophils are the most common leucocytes in most chelonians and crocodilians. Reptilian heterophils are typically of a round shape, the margins can be irregular and pseudopodia may occur. They show a size range from 10-23 μm. The nucleus is round to bean-shaped with densely clumped chromatin and located eccentrically. It may be lobed, especially in iguanas. The cytoplasm is colourless with eosinophilic, bright orange to salmon-coloured granules. The shape of the granules varies among species, although in chelonians they were generally described as fusiform in shape [Nardini et al., 2013].

The heterophils in *Gopherus agassizii* showed rod-shaped, eosinophilic granules in vast amounts of cytoplasm [Alleman et al., 1992]. In *A. horsfieldii* the granules were described as large and ovoid [Shadkhast et al., 2010] or as spindle-shaped with sharp poles [Knotková et al., 2002]. Heterophil size was characterized with a mean diameter of 13.2 μm in *T. hermanni* and 9.8 μm in *T. graeca* [Arikan et al., 2015]. For *A. horsfieldii* the size was reported as a diameter of 13.3 $\pm$ 0.5 μm [Knotková et al., 2002].

Eosinophils

Eosinophils of reptiles are generally described as spherical cells, with a size ranging from 9-20 μm. The nucleus is round to oval and positioned centrally or slightly peripheral in the cell. The cytoplasm is light blue and the large numbers of cytoplasmic granules are rounded and eosinophilic respectively salmon-coloured [Nardini et al., 2013]. Saint Girons [1970] differentiated two types of eosinophils, one with cylindrical and one with more spherical granules. Some authors described the nucleus as sometimes obscured by the cytoplasmic granules when these are packed densely [Saint Girons, 1970; Arikan et al., 2015].

The cytoplasmic granules in eosinophils of *A. horsfieldii* were characterised as darker and more rounded and red compared to heterophils [Knotková et al., 2002]. The size of eosinophils was reported with a mean diameter of 10.9 μm in *T. hermanni* and a mean

diameter of 12.5 μm in *T. graeca* [Arikan et al., 2015]. Other researchers reported a largest mean diameter of 14-19 μm in *T. graeca* [Saint Girons, 1970] and a diameter of 13.5 $\pm$ 0.8 μm in *A. horsfieldii* [Knotková et al., 2002].

Basophils

Basophils of reptiles are normally circular cells, the clear cytoplasm is tightly packed with basophilic, dark blue to purple granules. The slightly eccentric nucleus is round and often masked by the cytoplasmic granules. The size ranges from 7-20 μm [Nardini et al., 2013]. In *A. horsfieldii* the basophils were characterised with a diameter of 9.5 $\pm$ 0.6 μm [Knotková et al., 2002]. In another study a mean diameter of 11.9 μm in *T. hermanni* and a mean diameter of 10.9 μm in *T. graeca* was revealed [Arikan et al., 2015].

Lymphocytes

Reptilian lymphocytes are generally round to oval, however, the shape can also be irregular. They show a scant amount of faintly basophilic, pale blue cytoplasm. The cytoplasm is homogenous. The large, round to oval nucleus is positioned centrally or slightly eccentric with dark, heavily clumped chromatin. The nucleus to cytoplasm ratio is typically large. The size is described as 5-10 μm in small lymphocytes and 15 μm or more in large lymphocytes [Nardini et al., 2013]. The cytoplasm can show chromophobic vacuoles and few azurophilic granules [Saint Girons, 1970]. In *A. horsfieldii* the lymphocyte size was reported as a diameter of 5.7 $\pm$ 0.5 μm [Knotková et al., 2002]. Other authors characterised small lymphocytes with a mean diameter of 7.9 μm in *T. hermanni* and a mean diameter of 9.1 μm in *T. graeca*, while large lymphocytes were specified with a mean diameter of 11.7 μm in *T. hermanni* and a mean diameter of 12.1 μm in *T. graeca*. The large lymphocytes showed a higher amount of cytoplasm than the small lymphocytes where it was reduced to a thin margin along the nucleus [Arikan et al., 2015].

Monocytes

Monocytes are round to amoeboid in shape and the largest cells with a size range of 8-25 μm. The substantial cytoplasm appears pale blueish grey in colour and may seem slightly opaque or foamy. Phagocytised material and vacuoles occur, the size of the clear vacuoles depends on the activity [Heard et al., 2004]. Very fine eosinophilic or azurophilic granules can appear in the cytoplasm. The monocytic nucleus is of variable shape, it can be round, oval or lobed. In comparison with lymphocytes the nuclear chromatin is less condensed and stains paler [Nardini et al., 2013].

In *T. hermanni* the mean diameter was reported as 13.3 μm and in *T. graeca* as 12.0 μm. The cells were described as kidney-shaped with a dark purplish-blue nucleus and light grey cytoplasm [Arikan et al., 2015]. For *A. horsfieldii* a diameter of 15.5 $\pm$ 1.5 μm was reported and the shape was described as square-shaped with blue-grey spacious cytoplasm. The nuclei were characterised as large and quadratic with fine, pale chromatin [Knotková et al., 2002].

Alleman et al. [1992] reported two types of monocytes in the desert tortoise *Gopherus agassizii*: One appeared similar to mammalian monocytes, with an irregular nucleus and moderate amounts of basophilic cytoplasm that often contained clear vacuoles. The second type was described as similar to the first, but with a low to moderate amount of azurophilic granules within the cytoplasm. In this type, the nuclei were lobed to pleomorphic. The author further commented that the second type differed morphologically from the azurophils described in snakes or alligators and should best be accounted for as azurophilic monocytes.

Plasma cells

As described above, plasma cells are not often accounted for in literature. They are round to oval, with distinct borders and slightly larger than lymphocytes. The cytoplasm shows an intensely basophilic, deep blue colour with a perinuclear halo that represents the Golgi

complex. The nucleus is located eccentrically and round to oval-shaped with clumped chromatin. The size was described as slightly larger than lymphocytes, but not explicated further [Nardini et al., 2013]. Saint Girons [1970] also spoke of the perinuclear halo and described it as hyaloplasm. Other authors mentioned plasma cells as antibody-producing B–cells and differentiated them from lymphocytes by clumped chromatin in a “wagon wheel” shape [Heard et al., 2004].

Table 2.2. Morphological characterisation of reptilian blood cells as used in the present investigation.

Cell type	Characteristics
Erythrocytes	• large, elliptical cells • size: 14-23 μm long and 8-14 μm wide • eosinophilic cytoplasm • pyknotic nucleus, located centrally
Thrombocytes	• very small nucleus, round and condensed • clear to blue cytoplasm in small quantities, occasionally with scant cytoplasm • cytoplasm mostly with clear vacuoles, containing glycogen • granules mostly pink to purple • often mistaken for lymphocytes
Heterophils	• most common white blood cells in most chelonians and crocodilians • size: 10-23 μm • round cells, sometimes with irregular margins and pseudopodia • eosinophilic (bright orange to salmon-coloured) granules, shape and number varying in different species • colourless cytoplasm • nucleus central or slightly eccentric, round to bean-shaped
Eosinophils	• round cells • size: 9-20 μm • large numbers of eosinophilic round granules • light blue cytoplasm • nucleus round to oval, central or slightly eccentric

Table 2.2 – continued from previous page

Cell type	Characteristics
Basophils	• round cells • size: 7-20 μm • round nucleus, slightly eccentric, monolobed, often obscured by granules • clear cytoplasm, packed with round, basophilic (dark blue to purple) granules, variable in numbers
Lymphocytes	• round to oval cells, can be irregular-shaped • size: small lymphocytes 5-10 μm, large lymphocytes 15 μm or more • scant amount of slightly basophilic (pale blue) cytoplasm, homogeneous, lacks vacuoles or granules • nucleus central or slightly eccentric, large, round to oval-shaped with dark, heavily clumped chromatin • large nucleus to cytoplasm ratio
Monocytes	• round to amoeboid cells • size: 8-25 μm, largest cells • cytoplasm abundant, pale blue-grey, may be slightly opaque or foamy, vacuoles and phagocytized materials may be found • cytoplasm sometimes with fine eosinophilic/azurophilic granules • size of clear vacuoles in cytoplasm dependent on activity • nucleus variably shaped (round, oval or lobed), chromatin less condensed and paler in comparison to lymphocytes
Plasma cells	• round to oval cells • slightly larger than lymphocytes • distinct borders • intensely basophilic (deep blue) cytoplasm with a perinuclear halo (Golgi) • eccentric nucleus, round to oval with clumped chromatin

3. Material and methods

In order to investigate the influences of storage time and temperature of blood specimens on the blood smear with emphasis on the differential blood count and morphological changes in blood cells, a total of four Hermann's tortoises *(T. hermanni)* were sampled monthly over a period of four months. Each time, a blood sample was taken and aliquots were stored at different temperatures for different durations.

3.1. Animals

The blood samples were taken from July till October 2014. All samplings were carried out in the context of the propaedeutics classes for students of veterinary medicine at the Klinik für Vögel, Reptilien, Amphibien und Zierfische of the LMU Munich. The process of the blood sampling as described below was permitted by the German authorities and registered under the reference number Az. 55.2.1.54-2532.3-5-13; Anzeige § 10 TierSchG; "Übungen zur Untersuchung von Reptilien". The amounts of blood needed for the study were taken out of the samples before the rest was discarded. Four male individuals were used in this study with a weight range from 445-1085 g. The exact age of the animals was unknown, but all were assessed as adult.

3.1.1. Husbandry

The animals were kept at the Klinik für Vögel, Reptilien, Amphibien und Zierfische of the LMU Munich. An outside enclosure (Fig. 3.1) with a size of approximately 65 m^2 was provided for the outdoor husbandry of the animals from April till October/November with a freely accessible hotbed for weather protection and additional warmth during colder periods. The enclosure was fenced off using a low stone wall with blunted corners to avoid tortoises getting stuck. The soil was partly overgrown with grass and herbs, partly coarse rounded gravel. Larger rocks and pieces of wood were added to structure the space into different zones. Inside the hotbed, the ground was covered with a layer of hay and straw for feeding and burrowing. A water bowl was provided in the hotbed, shallow enough so that the smallest animal of the group could easily climb in and out without danger of drowning. A heat and UV-B lamp was available inside the hotbed for colder days and on hot days the roof was opened via an automated mechanism to enable circulating of fresh air and to prevent overheating. The tortoises mainly fed on fresh grass and herbs from the natural vegetal cover inside the enclosure during the summer; additional herbs were provided in irregular intervals. Seashells and cuttlebones were always available to the animals to cover their calcium needs.

3.1.2. Health monitoring

The animal population was free of herpesvirus and animals that were new to the group were quarantined separately for at least 4 weeks before joining the others in the outside enclosure. During quarantine, the animals were tested for herpesvirus infection using PCR according to VanDevanter et al. [1996] and multiple faeces samples were examined for endoparasites. The parasitological faecal examination was conducted using direct smears and a microscope. An individual animal was only turned outside with the group if it tested negative for herpesvirus and none or only non-pathogenic organisms were found in the faeces samples at least twice in a row. Visual monitoring of the animals was carried out daily, checking for any signs of impaired general condition.

Fig. 3.1. The outside enclosure for the Hermann's tortoises.

Before the actual beginning of the study, each tortoise was subjected to an extensive health check composed of a clinical examination (Fig. 3.2), a blood examination consisting of a chemical blood profile and an X-ray examination in three planes as well as a parasitologic faeces analysis to ensure the health of all involved animals. The protocols utilised for this health check and the clinical examination are enclosed in the appendix B.1.

The clinical examination comprised first the documentation of weight and body condition score. The body condition score was assessed using the scoring system shown in Table 3.1. Behaviour, integument, extremities, cloaca, mouth, nares, eyes and ears were assessed next, checking for species specific normal or abnormal attributes. Finally, the visceral cavity was palpated from the outside as far as possible.

Table 3.1. Determination of body condition score in Hermann's tortoises, according to: Lamberski [2013] and Calvert [2004], modified.

Body condition score	Characteristics
1: cachectic/emaciated	atrophied temporalis musculature, very prominent Crista sagittalis, cavernous eyes, reduced musculature on extremities
2: underfed	insufficiently distinct temporalis musculature, visible Crista sagittalis, slightly reduced musculature on extremities
3: normal	Crista sagittalis not visible but easily palpable, even surface of head, cervical and prefemoral skin appears thin
4: well fed	distinct temporalis musculature, surface of head slightly convex, Crista sagittalis not palpable, cervical and prefemoral subcutaneous fat vaguely recognisable, skin appears slightly thickened and protruding
5: obese	surface of head appears well rounded, axillar skin clearly thickened, pinchable fat, distinct subcutaneous fat on extremities and tail, cervical and prefemoral skin appears thickened, protruding

The chemical blood profile was established by an external laboratory (Synlab, Augsburg) and composed of alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, creatine kinase, triglycerides, urea, uric acid, sodium, potassium, calcium, phosphate, glucose, albumin and total protein.

The X-ray examination was performed using a Gierth HF400 *A* X-ray unit (Gierth X-Ray international, Riesa) with the software Agfa HealthCare NX (Agfa HealthCare, Greenville, USA) in a dorsoventral, laterolateral and craniocaudal projection to allow good assessment of the most important organ systems.

Fig. 3.2. Hermann's tortoise during the clinical examination.

3.2. Sampling

The first blood sampling in July was carried out on four tortoises. For the following three samplings, two of the initial four animals were used. The animals were always fixed by a second person with experience in handling of reptiles, to keep stress levels low in the animals. Before each blood sampling, the tortoises were subjected to a clinical examination as described above including weight monitoring as well as faeces analysis if a faeces sample could be obtained. The blood samplings were each carried out in July, August, September and October 2014. On colder days, the animals were placed in a tub with warm water below a heat and UV-B lamp for ∼20 minutes to allow them to reach the preferred body temperature of > 26 °C before collecting the blood specimen.

In both collection sites, if there was no blood flow, the needle was turned and retracted slightly to improve the positioning. The needle and syringe were always prepared beforehand by drawing up heparin-natrium 5000 IU/ml (B.Braun, Melsungen) and then discarding it, ensuring that a small amount of heparin remained in the conus of the needle. The obtained blood was transferred into a blood collection tube with lithium heparin. Before transfer, the needle was taken off the syringe. After filling the tube, it was pivoted three times.

The complete procedure was repeated with the same individuals after at least three weeks, to allow the animals abundant time to compensate the blood loss and recover sufficiently from possible overall strain.

3.2.1. Obtaining a blood specimen from the Vena coccygealis dorsalis

For the collecting of a blood sample from the Vena coccygealis dorsalis (Fig. 3.3), the tortoise was placed on the brink of a table, with its tail and hind legs projecting beyond the brink. The hind legs were locked into position with both hands by the fixing person to avoid kicking. This allowed the sampler to hold the tail stretched out straight with one hand and handling the syringe with the other hand, while the dorsal surface of the tail was facing towards the sampler. 1 ml syringes and 21 gauge x 25 mm needles were used. After cleaning and disinfecting the tail surface using kodan® (Schülke, Norderstedt, Art.-Nr. 104005), the hollow needle was inserted in a 45-90 ° angle along the dorsal median line, as far cranial as possible, with the cutting pointed ventrally. Subsequently the needle was pushed forward in a cranioventral direction, while maintaining a slight negative pressure. In case of making contact with a caudal vertebrae, the needle was retracted slightly and reoriented more cranially or caudally.

Sometimes it proved to be impossible to gather blood from the Vena coccygealis dorsalis and the specimen had to be collected from the Sinus venosus subcarapaxialis instead.

Fig. 3.3. Collecting blood from the Vena coccygealis dorsalis of a Hermann's tortoise.

3.2.2. Obtaining a blood specimen from the Sinus venosus subcarapaxialis

Alternatively to the Vena coccygealis dorsalis the Sinus venosus subcarapaxialis was used to collect the blood specimen. 21 gauge x 40 mm needles were used in this site. For the sampling, the fixing person restrained both front legs. Fixing the head is mostly not necessary in tortoises. Before inserting the needle it was bent slightly. The needle was inserted in the median, right below the transition between skin and carapace. A steep angle was maintained and the needle pushed forward in a dorsal direction. The needle was positioned correctly when the blood flowed freely.

3.3. Handling of specimens

After the sampling of the tortoises, five blood smears were produced directly from the blood collection tube with no delay. The rest of the specimen was divided into two equal portions and stored at 4 °C in the fridge or at room temperature. The room temperature specimens were stored inside a cupboard to avoid possible influences of direct sunlight. After storage for one hour, five blood smears were fabricated from each specimen. The process was repeated at 48 hours and 96 hours after the initial sampling. Before every spreading of a sample, the tube was pivoted three times. Of the five blood smears from each storage time and temperature group, the best three were each selected for further evaluation, yielding a total of 21 blood smears per animal and blood sample to be evaluated. The Table 3.2 provides a scheme of the samplings and the amount of smears produced per sampling and animal, while Figure 3.4 visualises the concept of the different storage times and temperatures for each sample. The first sampling in July 2014 provided four animals instead of the later two, as the blood samples from these additional animals were part of another study as well as the presented one.

Table 3.2. Blood sampling scheme.

1. sampling (July 2014)	4 animals (*T. hermanni* 1-4)	21 smears each – 84 smears total
2. sampling (August 2014)	2 animals (*T. hermanni* 1-2)	21 smears each – 42 smears total
3. sampling (September 2014)	2 animals (*T. hermanni* 1-2)	21 smears each – 42 smears total
4. sampling (October 2014)	2 animals (*T. hermanni* 1-2)	21 smears each – 42 smears total
		$\sum$ 210 smears

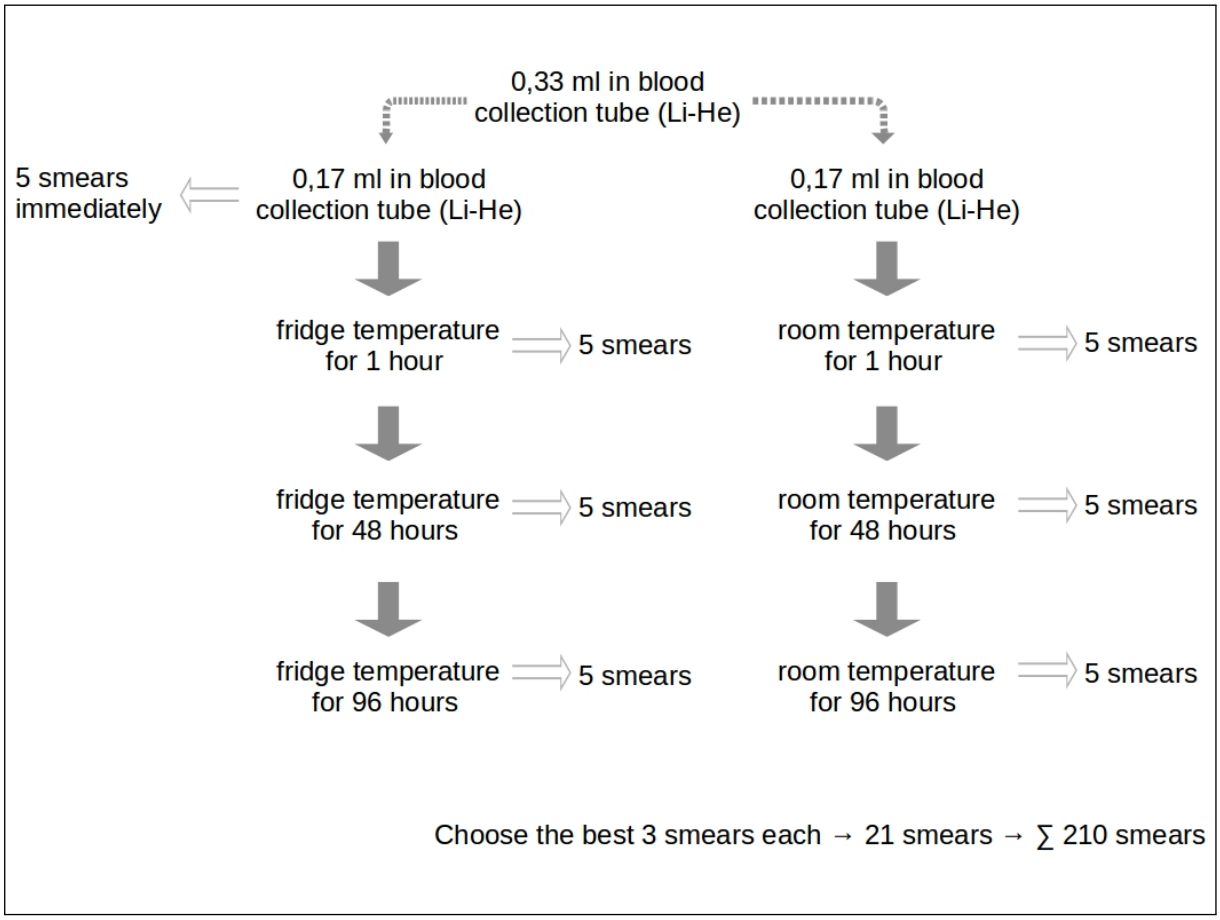

Fig. 3.4. Scheme for production of blood smears after differing storage time and temperature in each blood sample.

3.3.1. Production of blood smears

To produce the smears, a microlitre pipette was used to apply a small bubble-free drop of blood (2 μl) to a dust-free, dry glass microscope slide with a matted end for labelling. The slides were taken out of the package directly before they were needed. One pipette tip was used for five drops. The wedge smear technique (Fig. 3.5) was used to spread the blood drop over the slide.

The top slide had bevelled edges and rounded corners to reduce damage to the larger blood cells. It was held at an angle of $\sim$ 30-40 $^\circ$ and placed on the bottom slide. Then

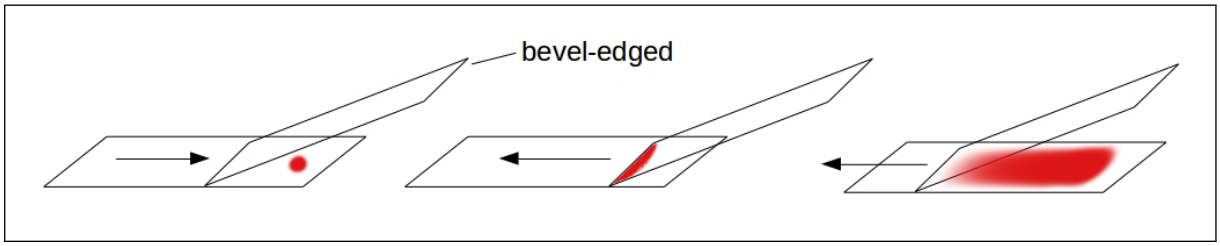

Fig. 3.5. Wedge smear technique, according to Pendl [2006], modified

it was pulled backwards until the edge met the blood drop. When the drop had spread out along the edge, the top slide was pushed forward in a smooth motion. All slides were left in a horizontal position for the smears to air-dry. If it was not possible to stain the smears immediately, they were fixed by submerging in methanol ($\geq$ 99,5 %, Carl Roth, Karlsruhe, Art.-Nr. 43.3) for 10 minutes.

3.3.2. Staining protocol

All smears were stained using a Pappenheim stain. The used staining protocol was a combination of those recommended by Mutschmann [2009] and Binder et al. [2012]. The Table 3.3 shows the exact steps of the staining protocol. The May-Grünwald solution used was the Merck (Darmstadt, Art.-Nr. 1.01424) May-Grünwald's eosine-methylene blue solution modified for microscopy, diluted 1:1 with demineralised water (pH 6.75-6.85). No filtration followed. The Giemsa solution used was the Merck (Darmstadt, Art.-Nr. 1.09204) Giemsa's azur eosin methylene blue solution, diluted 1:6 with demineralised water. Filtration with a pore size of 4-7 μm was performed before use. Demineralised water (pH 6.75-6.85) was used as buffering solution.

After the smears were completely air-dried, they were each mounted with a coverslip, using a mounting medium (Merckoglas®, Merck, Darmstadt, Art.-Nr. 1.03973), to allow permanent storage without damage or quality loss.

Table 3.3. Pappenheim staining protocol

1.	leave fresh smear to air-dry, stain at once preferably, otherwise fix in methanol for 10 min.	
2.	submerge completely in May-Grünwald solution	3 min.
3.	add same amount of buffer	2 min.
4.	dump the solution, no rinse	
5.	submerge completely in Giemsa solution	15 min.
6.	rinse with Aqua dest.	10 sec.
7.	submerge completely in buffer	4 min.
8.	submerge completely in fresh buffer	4 min.
9.	leave to air dry	

3.4. Evaluation of blood smears

All smears were evaluated according to a standardised protocol (see appendix B.2). Before beginning the actual evaluation, all necessary specifics were recorded on the form. The protocol consisted of a macroscopic and microscopic quality assessment, a differential blood count, a count of unidentifiable and/or damaged granulocytes, a thrombocyte count and the rating of the bacterial growth as well as the occurence of cytoplasmic vacuoles in the erythrocytes.

Even though the total leucocyte count is certainly a clinically relevant blood parameter in reptiles, it was not selected as a parameter for the evaluation of the influence of different storage conditions on the quality of the blood smears. The total leucocyte count in reptiles is rather strongly influenced by storage-independent factors such as the possible contamination of the blood samples with various amounts of lymph. Consequently, including blood samples from different sampling times in the evaluation would yield different results for the total leucocyte count. Therefore, the differential blood count was determined, which is not influenced by lymph contamination.

All smears were evaluated by the same person. The microscopic examination was performed in a meandering course, moving from one margin of the smear to the opposing margin. The magnification was 1000:1.

After evaluating the blood smears, 10 randomly selected smears were re-evaluated for verification. The values were compared with those from the first evaluation and due to deviations in the thrombocyte count, the first 37 smears were re-evaluated.

3.4.1. Microscope and camera equipment

The microscope used was a Leitz Aristoplan with a 100 x objective, suitable for oil immersion. The eyepiece was the Periplan GW 10 x, giving a total magnification of 1000:1. Immersion oil was always used. The camera used for the photographic documentation was the Leica WILD MPS 52V with the software Leica IM500 Image Manager (all in this paragraph: Leica Microsystems, Wetzlar).

3.4.2. Macroscopic quality assessment

The macroscopic quality assessment was the first step during the evaluation of a blood smear. First, the smear length was measured with a ruler. It should be at least 2.5 cm long, ideal was a length between 3-4 cm. The colour should appear pink in the thinner parts and purplish-blue in the thicker parts [Corrons et al., 2004]. The smear should become thinner towards the end and show a “tongue” rather than run out in flag-like ends. Documented as faults were a staged smear that appeared tremulous and holes in the blood film [Kraft et al., 2005]. All these criteria were confirmed or unconfirmed on the form with a Yes/No-system.

3.4.3. Differential blood count and thrombocyte count

For the differential blood count, a total of 100 leucocytes were counted and differentiated in each smear, using a manual counter. During the counting of 100 leucocytes, the thrombocyte count was also recorded.

3.4.4. Microscopic quality and other overall evaluations

For the microscopic quality, the overall impression of the smear was assessed after finishing the differential blood count. This included the assessment of the colour precipitation, which should be none to minimal and the uniform staining over the slide as well as the even distribution of the cells throughout the smear. These criteria were confirmed or unconfirmed using a Yes/No-system. Additionally, the bacterial growth and the cytoplasmic vacuoles in erythrocytes were assessed, rating the overall impression of the smear with a semiquantitative graded system, comprising the following gradings: - (none), + (sparse), ++ (medium) and +++ (plenty). The grading "sparse" was selected, when bacterial growth respectively cytoplasmic vacuoles in erythrocytes did not occur in every field of view and in low numbers from 1-5 per field of view. "Medium" was selected when bacteria respectively vacuoles occurred more frequently and in numbers from 6-10 per field of view. "Plenty" was selected when bacteria respectively vacuoles occurred frequently and in numbers from > 11 per field of view.

3.5. Statistical evaluation

The statistical evaluation was performed in cooperation with the Statistisches Beratungslabor (STABLAB) of the LMU Munich. The programming language R (Version 3.0.2) was used with the software RStudio (https://www.rstudio.com/). The evaluation of the data investigated the influence of the storage time and temperature on macroscopic and

microscopic quality, bacterial growth and cytoplasmic vacuoles in erythrocytes as well as leucocyte values.

3.5.1. Macroscopic and microscopic quality, bacterial growth and cytoplasmic vacuoles in erythrocytes

For the evaluation of the macroscopic and microscopic quality, bacterial growth and cytoplasmic vacuoles in erythrocytes, the median value was calculated for each triple value group from direct smears with no storage time and temperature. This median value was defined as the default value. The difference to this default value was calculated for all following values ascertained from blood smears made after storage of blood samples for defined points in time at room temperature or at the fridge. This difference was defined as the "quality indices" of the blood smear. The introduction of these quality indices allowed for a clear representation of the differences from each blood smear to the default value. The quality index was then scored using a scale including 2 (a difference of 2 to the default value), 1 (a difference of 1 to the default value), 0 (no difference to the default value), -1 (a difference of -1 to the default value), -2 (a difference of -2 to the default value) or -3 (a difference of -3 to the default value) By means of these quality indices, the data was examined for variation with increased storage time at the two temperatures.

A generalised linear model was then used to define the coefficients for the influence of the storage time and temperature on the quality index. Subsequently a generalised additive mixed model was used to investigate whether the individual animal and the month of blood sampling also influence the quality indices.

3.5.2. Leucocyte values

For the comparison of the differential blood count in the miscellaneous storage time and temperature combinations, the data for basophils, monocytes and plasma cells were pooled

as “rest”. Heterophil, eosinophil and lymphocyte values were weighted with 2 while the category “rest” was weighted with 1. This weighting was introduced to emphasise the distribution of the cell types with the largest percentages in the differential blood count for better comparison of the storage influences of the blood samples on the quality of the blood smear. Basophils, monocytes and plasma cells occurred in much lower percentages and were not considered representative for this comparison.

With a multinomial logit model, the alterations of the leucocyte values for the various storage time and temperature combinations was determined. Horizontal bar plots were produced for the illustration of the percentage of each cell type for each storage time and temperature combination.

4. Results

During the 4 months of the study, the tortoises were without pathological findings in the clinical and X-ray examinations. The chemical blood profiles from the initial examination were evaluated on the basis of the reference ranges found in several books and articles [Kölle, 2005; Heard et al., 2004; Mathes et al., 2006]. All animals were assessed as healthy, since in general only minimal deviations from the reference ranges occurred. Only the LDH values were distinctly low in all animals (range 54-220 U/l, reference range 708-1266 U/l [Kölle, 2005]). The results of the blood examinations are enclosed in the appendix C. No anthelminthic treatment was necessary in the course of the study. While collecting the blood samples from the Vena coccygealis dorsalis, two of the four animals were sampled without problems throughout the four months of the study. One animal had to be punctured more than once in every month. The last animal had to be punctured more than once in the first three months and was sampled without problems in the last month. In one animal the sampling from the Sinus venosus subcarapaxialis was necessary once as it was not possible to obtain a blood sample from the Vena coccygealis dorsalis. Complications such as possible haemorrhage from the lungs, did not occur during sampling at this site.

During the microscopic evaluation of the blood smears, three smears were not fully evaluable, as it proved impossible to find 100 leucocytes for differentiation. All of these smears were from the same blood sample, stored at room temperature for 96 h. In one smear 67 leucocytes were found and differentiated, these results were included in the statistical analyses, as it was still possible to determine a conclusive percentage distribution from this counting. The other two smears were excluded, as only 23 respectively 17 leucocytes were

found and differentiated and this count was too low to establish a conclusive percentage distribution.

When re-evaluating 10 randomly selected blood smears and comparing the obtained values to those from the first evaluation, it became apparent that the thrombocyte count was too low at the beginning of the evaluations. Therefore, the first 37 blood smears were re-evaluated and the thrombocyte count stabilised itself at about the 35th smear of 210. The values of the leucocytes and the other evaluated criteria did not deviate much from those obtained in the first evaluation.

4.1. Statistical analyses

4.1.1. Macroscopic quality, microscopic quality, bacterial growth and cytoplasmic vacuoles in erythrocytes

The relation of storage time and macroscopic/microscopic quality, bacterial growth and cytoplasmic vacuoles in erythrocytes was examined for both storage temperatures. To examine the progression of these attributes during prolonged storage time, the median of the individual parameter was determined in the smears produced immediately after venipuncture and defined as the default value. For the values ascertained from blood smears made after storage of blood samples for defined points in time at room temperature or at the fridge, the difference to this default value was calculated. This difference was defined as "quality index" of the blood smear and the quality indices were scored using a scale of 2 to -3, as described in the following listing. Further calculations were carried out using only these differences to the default value for the purpose of standardisation. All deviations occuring during any kind of storage of a blood sample were therefore compared to the individual condition of exactly the same blood sample directly after venipuncture. Therefore it was not considered expedient to indicate the median and the variation coefficient in the table of results (Table A.1). All individual values are certainly presented in Table A.1.

Interpretation of the tables:

- Quality index = 2 means an improvement of the attribute by 2 compared to the default value.
- Quality index = 1 means an improvement of the attribute by 1 compared to the default value.
- Quality index = 0 means no change in the attribute compared to the default value.
- Quality index = -1 means a decline of the attribute by 1 compared to the default value.
- Quality index = -2 means a decline of the attribute by 2 compared to the default value.
- Quality index = -3 means a decline of the attribute by 3 compared to the default value.

Note: This quality index is **not** in any way related to the terms macroscopic/microscopic quality.

The following tables, one for each attribute, display the percentage count of smears showing the particular quality index for each storage time/temperature combination.

Progression of the macroscopic quality

The assessment of the macroscopic quality was the first step of the evaluation of a blood smear and comprised of six criteria. The smear length and colouring were evaluated as well as the thickness of the layer and the end morphology of the smear, which should be running out in a tongue rather than flag-like. The smear was also checked for holes

and a tremulous course. All these criteria were assessed with a Yes/No-system and then summarised as the macroscopic quality, ranging from 1 (good) to 3 (unsatisfactory).

As shown in Table 4.1, the macroscopic quality was not strongly influenced by the storage time and storage temperature of the blood specimens. There were no large differences in the percental distribution of the smears to the quality indices between storage for 1 h and 96 h in both temperatures.

After a storage for 48 h, the macroscopic quality improved by 1 in 16.66 % of the smears from fridge-temperature specimens and in 50 % of the smears from room-temperature specimens. For the fridge temperature, 50 % of the smears showed no change in the macroscopic quality as opposed to 30 % for the room temperature. While 30 % of the smears from fridge-temperature specimens declined in quality by 1, for the room-temperature specimens it was 10 %. For the other storage time and temperature combinations the differences were less distinct, ranging from 3.34 % - 13.34 %.

Table 4.1. Macroscopic quality: Percentages of blood smears with quality indices of 1 to -2 after storage at fridge or room temperature for 1 h, 48 h or 96 h; n = number of animal blood samplings included for determination of the values. The median values of three smears from each sampling were used for calculation. For 96 h at room temperature, two smears were not fully evaluable and therefore excluded from the results.

			Quality index			
		n	1	0	-1	-2
Fridge temperature	1 h	10	30	43.34	20	6.66
	48 h	10	16.66	50	30	3.34
	96 h	10	20	50	30	0
Room temperature	1 h	10	43.34	30	26.66	0
	48 h	10	50	30	10	10
	96 h	10	23.34	53.34	16.66	6.66

Progression of the microscopic quality

The assessment of the microscopic quality of a blood smear comprised three criteria. The colour precipitation, the evenness of cell distribution and the uniform staining of the smear were evaluated with a Yes/No-system and then summarised as the microscopic quality, with a range from 1 (good) to 3 (unsatisfactory).

The quality indices calculated based on comparisons with the default values determined for smears produced immediately after venipuncture are presented in Table 4.2. With increasing storage time the microscopic quality declined clearly in both temperatures, with the room-temperature specimens at 96 h showing a change for the worse to a quality index of -1 in 43.34 % of the blood smears and to a quality index of -2 in 33.34 % of the blood smears. In comparison to the fridge temperature 23.34 % more of the smears showed a strong decline to a quality index of -2 after 96 h. After 1 h and 48 h there were smaller differences between the two storage temperatures. For the room temperature, more deviations in the quality index occurred already after 1 h storage compared to the fridge temperature. No changes to the quality index were more frequently found in the fridge-temperature samples than in the room-temperature samples for all storage times.

Table 4.2. Microscopic quality: Percentages of blood smears with quality indices of 2 to -2 after storage at fridge or room temperature for 1 h, 48 h or 96 h; n = number of animal blood samplings included for determination of the values. The median values of three smears from each sampling were used for calculation. For 96 h at room temperature, two smears were not fully evaluable and therefore excluded from the results.

			Quality index				
		n	2	1	0	-1	-2
Fridge temperature	1 h	10	0	16.66	53.34	26.66	3.34
	48 h	10	3.34	6.66	26.66	43.34	20
	96 h	10	0	6.66	36.68	46.66	10
Room temperature	1 h	10	3.34	16.66	40	36.66	3.34
	48 h	10	0	13.32	23.34	43.34	20
	96 h	10	0	0	23.32	43.34	33.34

Progression of the bacterial growth

The bacterial growth was assessed using a rating system ranging from - (none) to +++ (plenty) and based on a comparison of these ratings with the default value, the quality indices shown in Table 4.3 were calculated.

All specimens from both temperatures showed no changes in the quality index after 1 h. At 48 h 96.66 % of the fridge-temperature specimens remained unchanged while 3.34 % declined to a quality index of -1. In contrast to this, 76.66 % of the room-temperature smears retained the same quality level while 13.34 % were reduced to a quality index of -1 and 10 % declined to a quality index of -2. After 96 h the fridge-temperature specimens showed 90 % of the smears with no changes in the quality index and 10 % were declined to a quality index of -1. Of the room-temperature specimens, 10 % were unchanged and 33.34 % were decreased to a quality index of -1 while 6.66 % showed a decrease to -2 and 50 % were reduced to a quality index of -3 (Table 4.3).

Table 4.3. Bacterial growth: Percentages of blood smears with quality indices of 0 to -3 after storage at fridge or room temperature for 1 h, 48 h or 96 h; n = number of animal blood samplings included for determination of the values. The median values of three smears from each sampling were used for calculation. For 96 h at room temperature, two smears were not fully evaluable and therefore excluded from the results.

			Quality index			
		n	0	-1	-2	-3
Fridge temperature	1 h	10	100	0	0	0
	48 h	10	96.66	3.34	0	0
	96 h	10	90	10	0	0
Room temperature	1 h	10	100	0	0	0
	48 h	10	76.66	13.34	10	0
	96 h	10	10	33.34	6.66	50

Progression of the cytoplasmic vacuoles in erythrocytes

The cytoplasmic vacuoles in erythrocytes were assessed using a rating system ranging from - (none) to +++ (plenty).

As shown in Table 4.4, after 1 h storage there were no changes in the quality index in both storage temperatures. 96.66 % of the fridge temperature specimens remained unchanged in quality at 48 h while 3.34 % decreased to a quality index of -1. The room-temperature specimens on the other hand showed a greater distribution at 48 h with 43.34 % remaining unchanged, 50 % declined to a quality index of -1 and 6.66 % declined to a quality index of -2. At 96 h the fridge-temperature specimens showed 30 % unchanged blood smears while 30 % were declined to a quality index of -1, 23.34 % to a quality index of -2 and 16.66 % to a quality index of -3. In comparison to this, the room-temperature specimens showed 3.34 % unchanged smears, 23.34 % were declined to a quality index of -1, 46.66 % were decreased to a quality index of -2 and 26.66 % were decreased to a quality index of -3.

Table 4.4. Cytoplasmic vacuoles in erythrocytes: Percentages of blood smears with quality indices of 0 to -3 after storage at fridge or room temperature for 1 h, 48 h or 96 h; n = number of animal blood samplings included for determination of the values. The median values of three smears from each sampling were used for calculation. For 96 h at room temperature, two smears were not fully evaluable and therefore excluded from the results.

			Quality index			
		n	0	-1	-2	-3
Fridge temperature	1 h	10	100	0	0	0
	48 h	10	96.66	3.34	0	0
	96 h	10	30	30	23.34	16.66
Room temperature	1 h	10	100	0	0	0
	48 h	10	43.34	50	6.66	0
	96 h	10	3.34	23.34	46.66	26.66

Influence of the month of blood sampling and the single animal on the quality indices

When investigating the distribution of the quality indices for the month of blood sampling and the single animal with a generalised additive mixed model, a statistical spread was found among the months of blood sampling. Among the animals a variance was then found within the months. The standard deviation for the influence of the month of blood sampling was 0.13 and when investigating the influence of the individual animal within the month of blood sampling, the standard deviation was 0.33.

This means, aside from the different storage conditions, the month of blood sampling and the individual animal had an influence on the distribution of the quality indices for all four attributes specified above.

4.1.2. Leucocyte values

The percental distribution of each cell type for each storage time/temperature combination is presented in the Table 4.5. The results are also visualised in two horizontal bar plots, one for the storage at fridge temperature (Fig. 4.1) and one for the storage at room temperature (Fig. 4.2).

Table 4.5. Percentage of eosinophils, lymphocytes, heterophils and other leukocytes (rest) in smears produced from blood stored at different temperature-time-combinations (Leucocyte values in %, rest = basophils, monocytes and plasma cells); n = number of animal blood samplings included for determination of the values. The median values of three smears from each sampling were used for calculation. For 96 h at room temperature, two smears were not fully evaluable and therefore excluded from the results.

		n	Eosinophils	Lymphocytes	Heterophils	Rest
	No storage	10	10	58	29	3
Fridge temperature	1 h	10	12	58	28	2
	48 h	10	16	56	25	3
	96 h	10	15	59	22	4
Room temperature	1 h	10	13	53	31	3
	48 h	10	19	58	20	3
	96 h	10	14	78	5	3

For the fridge temperature, the percentages of all cell types showed no distinct changes for all storage times. For the room temperature, the heterophil count remained all but the same before declining noticeably with increasing storage time, beginning at 48 h. The lymphocyte count was increased accordingly after 96 h. After 48 h the eosinophil count was increased slightly in comparison to the other storage times. Aside from this exception, the eosinophil count at room temperature was less influenced by storage time than heterophil and lymphocyte count. The group "rest" consisted of basophils, monocytes and plasma cells and did not show remarkable deviations in any of the storage time/temperature combinations. This group also continuously represented the smallest percentage of the differential blood count.

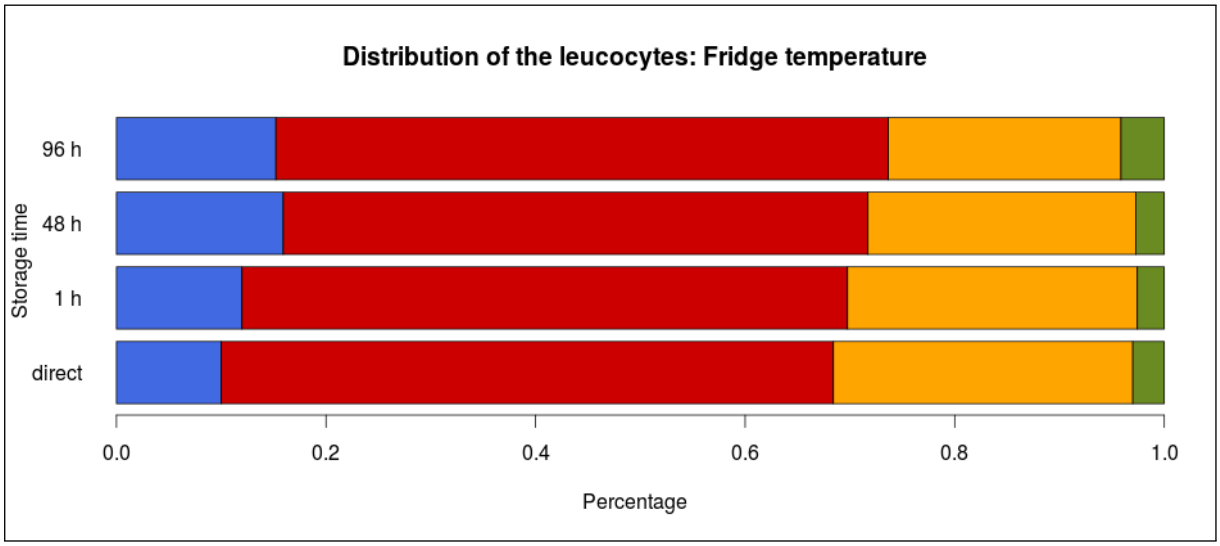

Fig. 4.1. Distribution of leucocytes for fridge storage.
Blue = eosinophils, red = lymphocytes, yellow = heterophils, green = rest (Basophils, monocytes and plasma cells pooled)

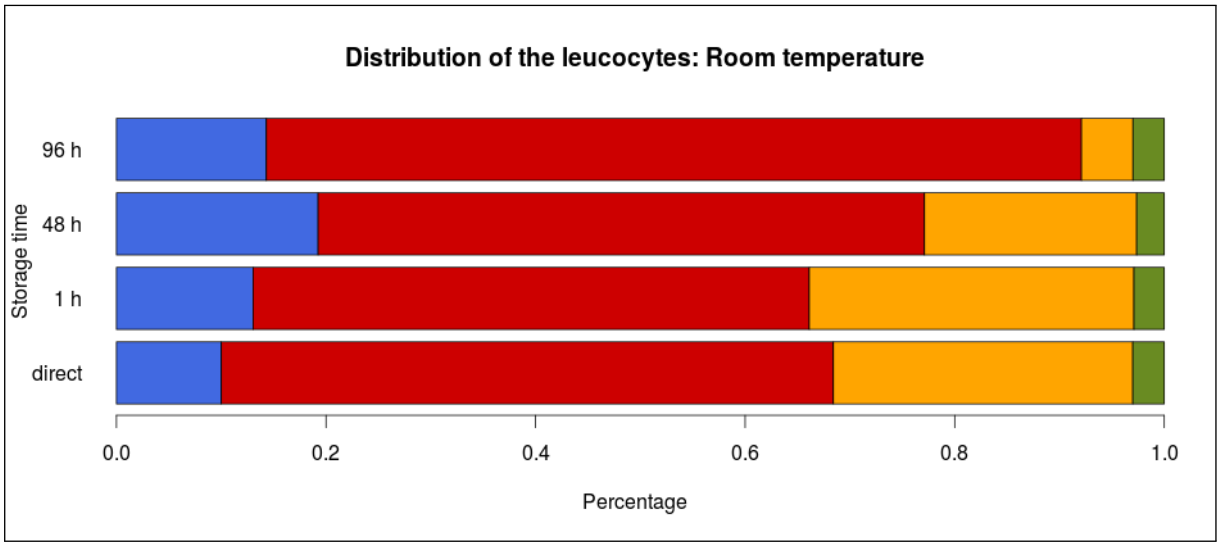

Fig. 4.2. Distribution of leucocytes for room storage.
Blue = eosinophils, red = lymphocytes, yellow = heterophils, green = rest (Basophils, monocytes and plasma cells pooled)

4.2. Morphological changes in blood cells

Examples of morphological changes in the blood cells during storage were documented in a semiquantitative way for each leucocyte cell type, excluding the plasma cells, as only very few (< 10) cells could definitely be identified as plasma cells during the study. The terms “often” and “frequently” hereby represent a count of 141-210 smears, “in some cases”, “sometimes” and “occasionally” refer to a range of 71-140 smears and “few” refers to > 70 smears. In either case, this semiquantitative definition is related to each cell type separately. Changes in thrombocytes were also documented. The blood cells displayed very different changes in morphology during storage, so the examples presented here should only be considered as such.

A strong bacterial growth and cytoplasmic vacuoles in erythrocytes were often found in smears from samples stored at room temperature for 96 h (Fig. 4.3). However, this was not always the case (for example Fig. 4.16, 4.25 and 4.49) and the photographic documentation of the leucocytes also serves as an exemplary presentation of the differing progression of the erythrocyte appearance and overall blood smear quality.

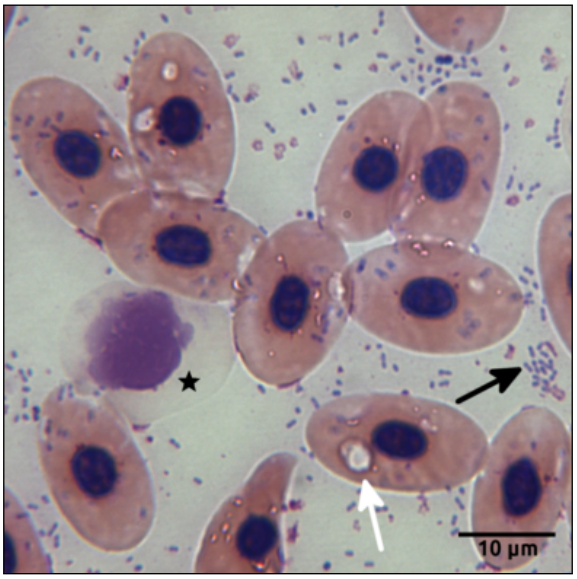

Fig. 4.3. Bacterial growth (black arrow) and cytoplasmic vacuoles (white arrow) in erythrocytes; smear produced from blood after storage of 96 h at room temperature. Also note lysed erythrocyte (black star). Pappenheim stain, 1000x.

The morphology of the erythrocytes progressed differently during prolonged storage at both temperatures. Lysed erythrocytes occurred occasionally in smears produced from blood after storage of 48 h at room temperature (for example Fig. 4.12 and 4.47). After storage of the blood sample for 96 h at fridge temperature, the erythrocytes showed a darker colouring in some smears (for example Fig. 4.6). A deterioration of the erythrocytes was observed often and in differing varieties, for example with a strong, bubbly vacuolisation of the cytoplasm (Fig. 4.22), disintegrating margins (Fig. 4.24) or a pale cytoplasm (Fig. 4.15, 4.26, 4.40 and 4.57). In some smears produced from blood stored at room temperature for 96 h, the erythrocytes showed a deterioration of the cytoplasm, such as in Fig. 4.16, 4.34, 4.42, 4.50 and 4.60. This occasionally corresponded with a crumpled appearance of the cytoplasm (for example Fig. 4.23, 4.33, 4.49 and 4.58). A loss of the typical oval shape, pale nuclei and disintegrating cytoplasm were also observed in some cases (Fig. 4.25). Vacuoles appeared either in a small size and located

marginally in the cytoplasm (Fig. 4.41) or in a larger size anywhere in the cytoplasm (Fig. 4.34).

4.2.1. Heterophils and eosinophils

Heterophils normally appeared as large, more or less rounded cells with orange to salmon-coloured granules, which were fusiform in shape. In comparison to the heterophils, the eosinophils were mostly easy to distinguish (Fig. 4.4). Eosinophils usually also presented themselves as large cells, similar to heterophils in shape. The shape and colour of eosinophilic granules differed from heterophilic granules. Normal eosinophilic granules were rounded and showed a pale pink colouring. The nuclei in both granulocyte types also appeared similar, of a dark purple colour, round to bean-shaped and mostly located eccentrically in the cell. Clumped chromatin was recognisable in the nuclei (for example Fig. 4.8ff., 4.17ff.). Heterophils were sometimes binucleated or showed bilobed nuclei.

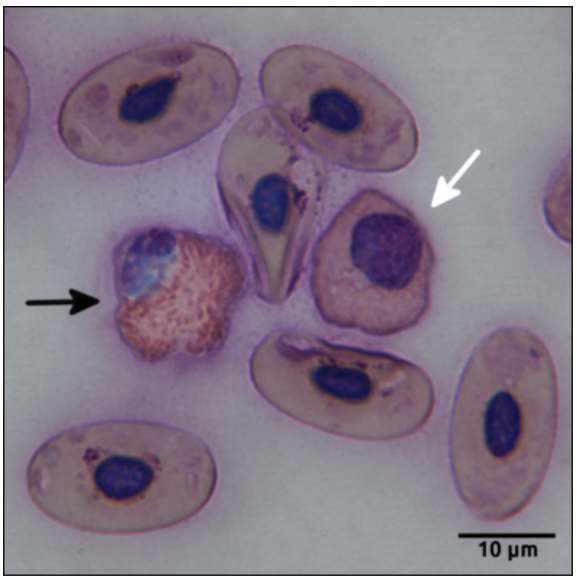

Fig. 4.4. Heterophil (black arrow) and eosinophil (white arrow); smear produced from blood after storage of 1 h at fridge temperature. Pappenheim stain, 1000x.

In heterophils, the cytoplasmic granules often became less distinct with prolonged storage (Fig. 4.11ff.). The granules conglomerated completely in some, but not in all cases (Fig. 4.14 and 4.16). Fig. 4.15 shows a heterophil with lysed cytoplasm and granules after storage of 96 h at fridge temperature, but the dark stippling of the granules is still recognisable. On the other hand, in the smear from a blood specimen stored at room temperature for 96 h, the heterophilic granules have completely merged and form a homogenous salmon-coloured area (Fig. 4.14).

The eosinophilic granules tended to darken in colour during storage, which became especially noticeable in samples stored for 96 h. The storage temperature did not seem to have an influence on this, darker granules were seen in both temperatures (Fig. 4.23 and 4.26). A blackberry-like appearance of the eosinophils was found occasionally (Fig. 4.23).

The chromatin clumping in the nuclei, which was very distinct in heterophils and eosinophils of smears produced from blood immediately after venipuncture, became less detailed with prolonged storage in both cell types (for example Fig. 4.14ff. and 4.21ff.) until the nuclei were a homogenous purple area.

With prolonged storage it became more difficult to differentiate heterophils and eosinophils. After 48 h storage, the colour of the cytoplasmic granules appeared more similar in heterophils and eosinophils (Fig. 4.5). Due to the rather distinct demarcation of the eosinophilic granules from each other, the differentiation between eosinophil and heterophil was still clear. After storage for 96 h, heterophils and eosinophils often showed a similarly dark shading of the cytoplasmic granules and were difficult to differentiate from each other. The most important evidence for differentiation in these cases was provided by the colour of the cytoplasmic granules. Heterophils often appeared slightly more orangish and intense in colour, while eosinophils were of a dark pink and duller in colour (Fig. 4.6).

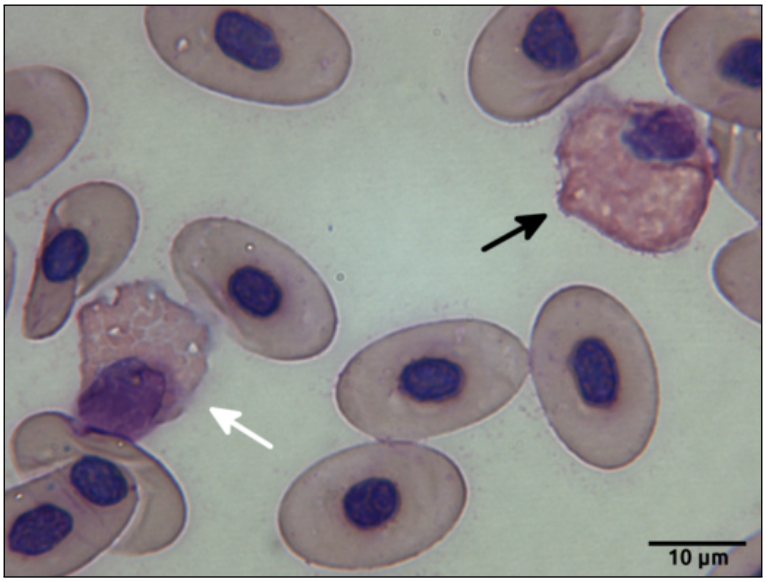

Fig. 4.5. Heterophil (black arrow) and eosinophil (white arrow); smear produced from blood after storage of 48 h at fridge temperature. Pappenheim stain, 1000x.

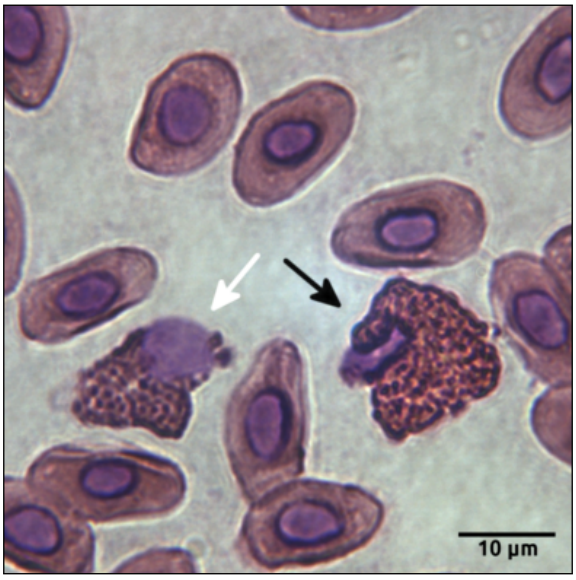

Fig. 4.6. Heterophil (black arrow) and eosinophil (white arrow); smear produced from blood after storage of 96 h at fridge temperature. Pappenheim stain, 1000x.

4.2.2. Lymphocytes and thrombocytes

While large lymphocytes and thrombocytes were readily distinguished from each other, small lymphocytes and thrombocytes frequently presented difficulties when it came to differentiation.

Lymphocytes were basically round, but their shape also varied due to molding around the adjacent cells. The nuclei were large and showed clumped chromatin of a purple colour. Small lymphocytes displayed only a narrow margin of cytoplasm, while large lymphocytes had more cytoplasm in relation to nucleus size. The cytoplasm was a light blue-gray colour.

The thrombocytes were often the smallest cells and presented a great variation in appearance. The shape ranged from round to irregular, elliptical, bean-shaped and spindle-shaped. Thrombocytic nuclei were large and showed condensed chromatin, which appeared dark purple. The cytoplasm was clear to light purplish-blue, often depending on the overall staining of the blood smear. Thrombocytes were most easily identified when they formed cell clusters, as lymphocytes do not aggregate as such, which also provided a reference for the appearance of thrombocytes in the blood smear (Fig. 4.53). Two different forms of thrombocytes were found during the evaluation. The more common type showed various shapes and a dark purple nucleus with condensed chromatin. The clear to light purple cytoplasm appeared wrapped around the nucleus in irregular shapes (Fig. 4.51, 4.54 and 4.56). The second type was more uniform, elliptical, with elliptical nuclei and light purple cytoplasm. These cells were larger compared to the other type (Fig. 4.52).

In thrombocytes as well as lymphocytes, the cytoplasm initially did not show vacuoles. With prolonged storage, small cytoplasmic vacuoles appeared, especially at room temperature (Fig. 4.39 and 4.56). Also, the cells showed an increasing loss of detail (Fig. 4.40ff. and 4.57ff.). Depending on the appearance of the surrounding erythrocytes, especially the thrombocytes could become very difficult to differentiate from the erythrocyte nuclei or colour stains (Fig. 4.57).

4.2.3. Basophils

Basophils were round cells with very dark purple, small cytoplasmic granules. The granules were packed extremely tight and mostly obscured the nucleus. Sometimes degranulated basophils were found, which showed a clear cytoplasm with less granules. During storage, basophils did not change much during 1 h and 48 h storage in both temperatures (Fig. 4.27ff.). After storage of 96 h at fridge temperature, they sometimes showed an even darker, almost black, purple colouring (Fig. 4.32). After 96 h at room temperature, in some basophils the nucleus was distinctly visible as the granules had retreated. The nucleus showed a pale purple colour in these cells (Fig. 4.33). Few basophils showed protruding cytoplasmic and granular appendage, similar to pseudopodia (Fig. 4.34).

4.2.4. Monocytes

Monocytes were among the largest cells, with a rounded shape and a large nucleus-cytoplasm ratio. The nuclei showed an intensely purple colour with clumped chromatin. The cytoplasm was variable in appearance. The colour ranged from pale blue-grey to deep purplish blue. Sometimes a slight granulation with fine granules was present (Fig. 4.45 and 4.46). Cytoplasmic vacuoles (Fig. 4.44) were seen often and seemed to increase with prolonged storage at both temperatures (Fig. 4.47ff.). With storage at room temperature for 96 h, the clumped chromatin in the nuclei became less distinct and the nuclei often appeared as a homogenous area of purple colour (Fig. 4.49 and 4.50).

4.2.5. Plasma cells

Only very few (< 10) plasma cells (Fig. 4.7) could be definitely identified during the evaluations. Therefore their number does not appear in the differential blood counts (Table A.1) and a complete photographic documentation for all storage time and temperature

combinations as in the other cell types could not be established. These cells were larger than lymphocytes and rounded in shape. The purple nucleus showed distinct chromatin clumping and was located eccentrically. The cytoplasm was a deep blue, with a lighter coloured area close to the center of the cell and adjacent to the nucleus. This lighter colouring faded into the surrounding blue colour of the cytoplasm without a distinct margin.

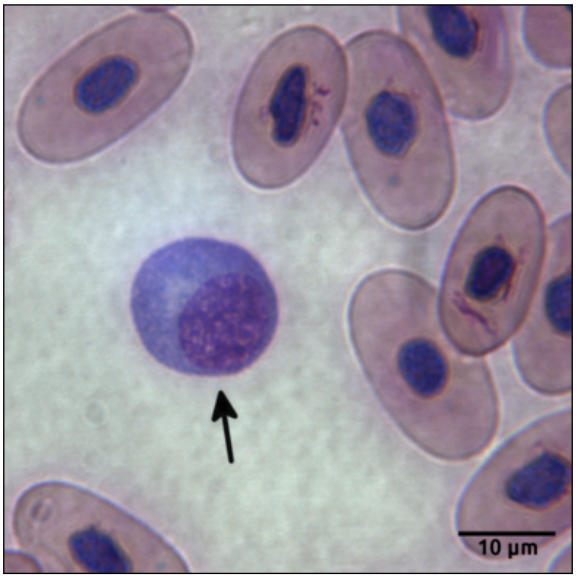

Fig. 4.7. Plasma cell (black arrow); smear produced directly after blood sampling. Pappenheim stain, 1000x.

4.3. Microscopic photos

In the following, the photographic documentation of morphological changes in the blood cells is presented. All photos were taken by the author herself. Since the changes in morphology developed quite differently, these photos should strictly be considered as examples of possible changes. Annotations are provided underneath each image. All photos were taken from Pappenheim stained smears, at a magnification of 1000:1. The process is described in the chapter 3: Material and methods.

4.3.1. Heterophils

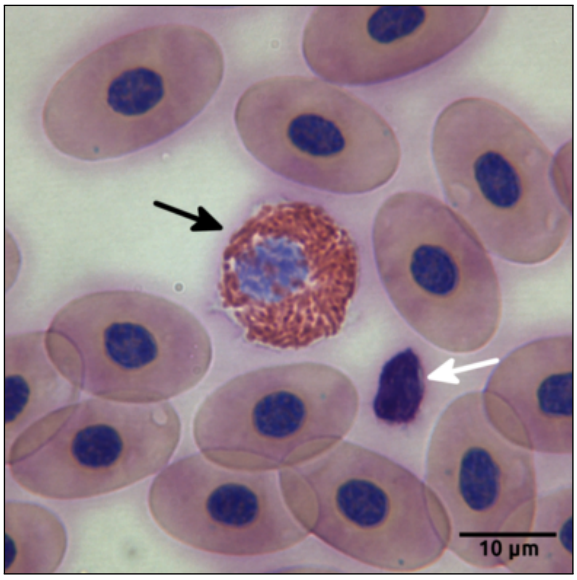

Fig. 4.8. Heterophil (black arrow); smear produced directly after blood sampling. Normal appearance with salmon-coloured, fusiform, tightly packed granules. The other cell is a thrombocyte (white arrow). Pappenheim stain, 1000x.

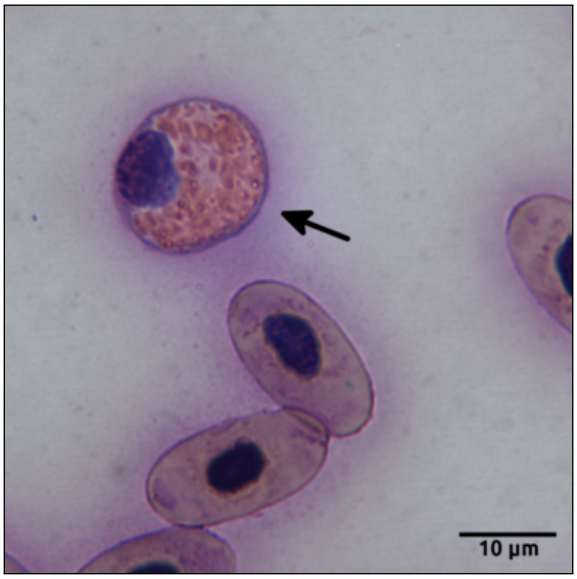

Fig. 4.9. Heterophil (black arrow); smear produced from blood after storage of 1 h at fridge temperature. Granules show normal colour, but appear less packed. Note the purple margin around the cell, which appeared occasionally on heterophils and is probably a result of staining. Pappenheim stain, 1000x.

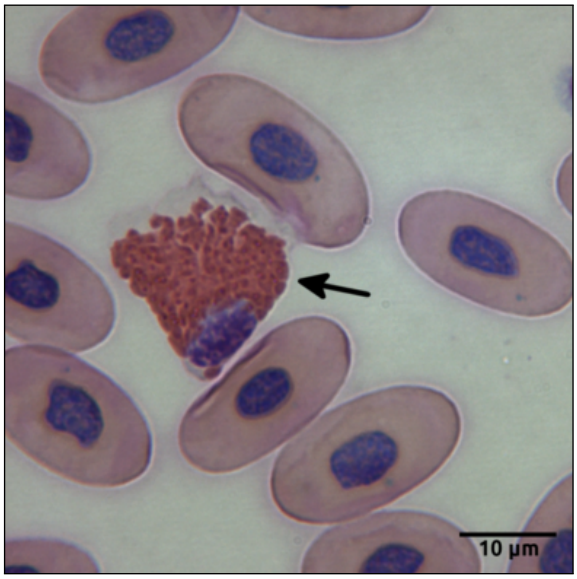

Fig. 4.10. Heterophil (black arrow); smear produced from blood after storage of 1 h at room temperature. Beginning conglomeration of the granules, but normal colouring. Pappenheim stain, 1000x.

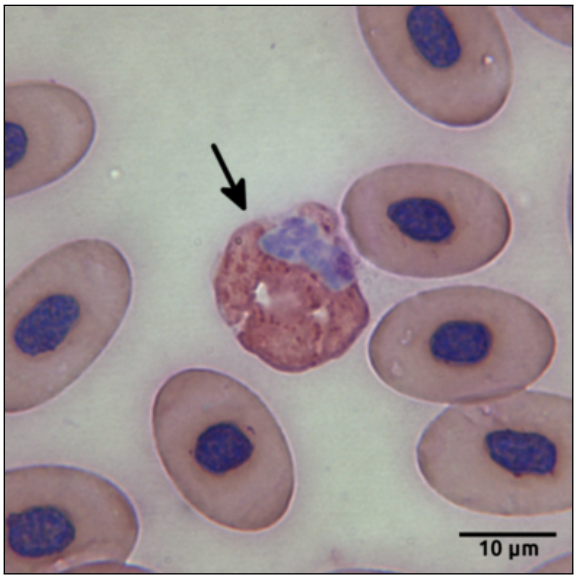

Fig. 4.11. Heterophil (black arrow); smear produced from blood after storage of 48 h at fridge temperature. Barely any single granules recognisable. Pappenheim stain, 1000x.

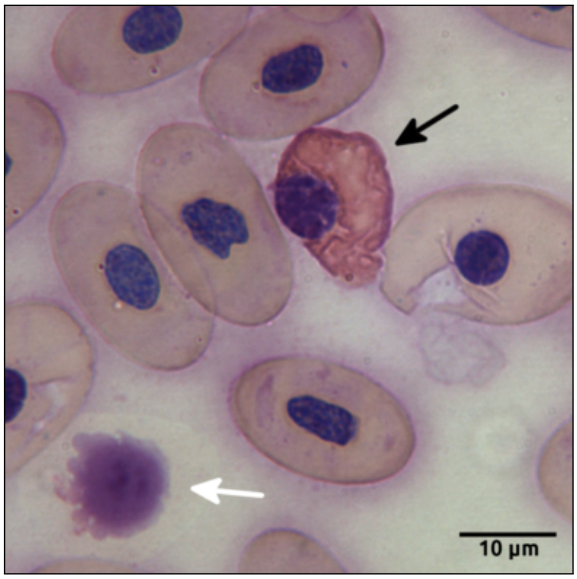

Fig. 4.12. Heterophil (black arrow); smear produced from blood after storage of 48 h at room temperature. No granules recognisable. Also note lysed erythrocyte (white arrow). Pappenheim stain, 1000x.

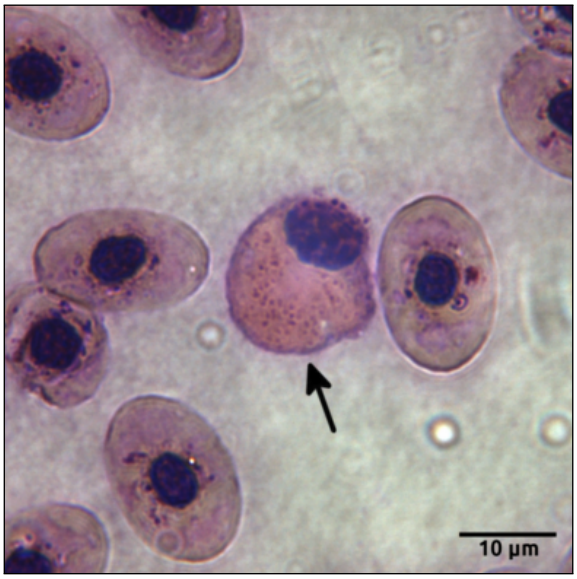

Fig. 4.13. Heterophil (black arrow); smear produced from blood after storage of 96 h at fridge temperature #1. No granules recognisable, but darker stippling is visible. Note the deterioration of the surrounding erythrocytes. Pappenheim stain, 1000x.

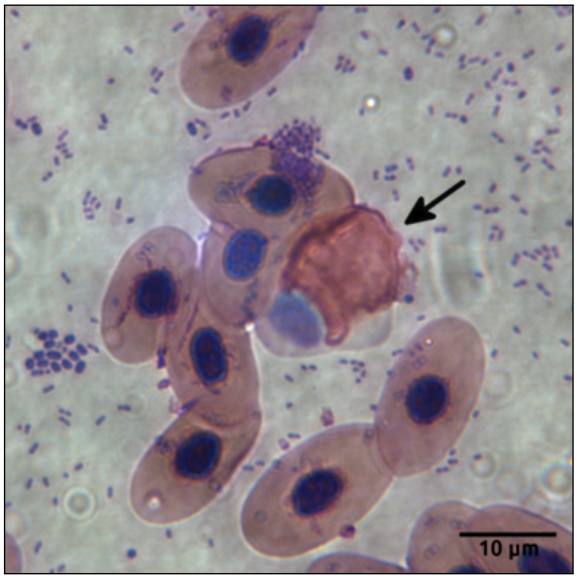

Fig. 4.14. Heterophil (black arrow); smear produced from blood after storage of 96 h at room temperature #1. No granules recognisable, homogenous salmon-coloured area. Note the strong bacterial growth. Pappenheim stain, 1000x.

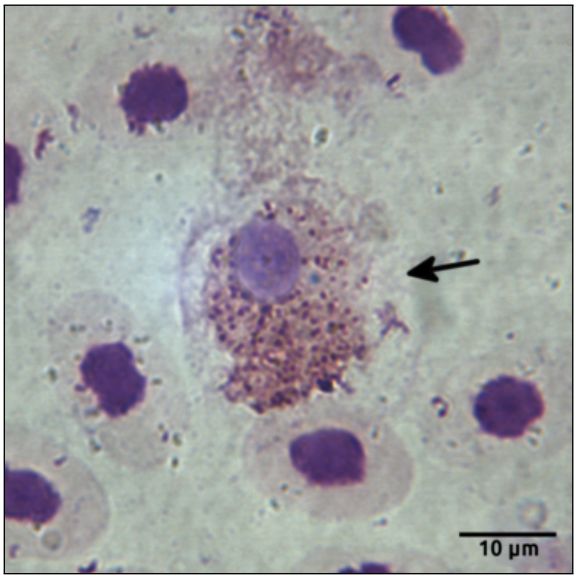

Fig. 4.15. Heterophil (black arrow); smear produced from blood after storage of 96 h at fridge temperature #2. Lysed cytoplasm, no single granules recognisable, but a distinct dark stippling. Note also the pale colour of erythrocyte cytoplasm. Pappenheim stain, 1000x.

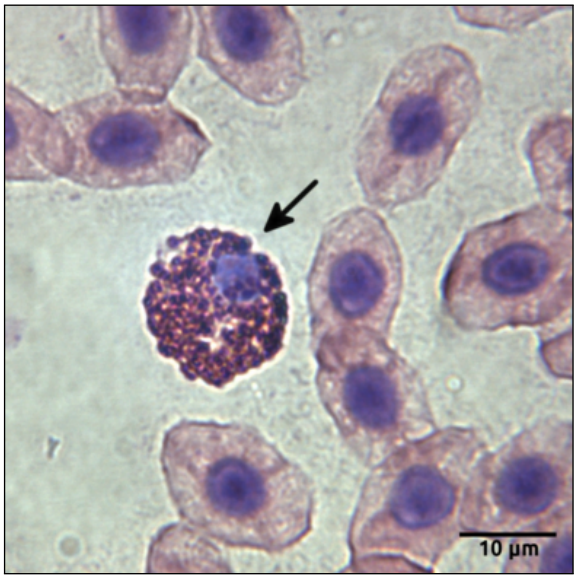

Fig. 4.16. Heterophil (black arrow); smear produced from blood after storage of 96 h at room temperature #2. Granules preserved, but with darker colour and more rounded shape. No bacterial growth despite long storage at room temperature. Note the deteriorating cytoplasm of the erythrocytes. Pappenheim stain, 1000x.

4.3.2. Eosinophils

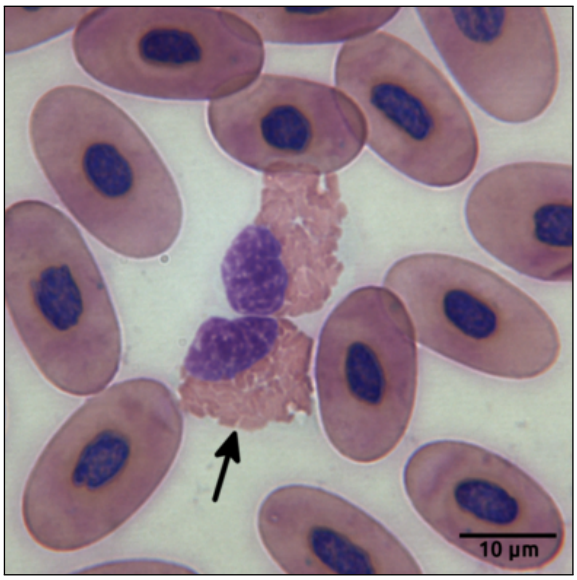

Fig. 4.17. Eosinophils (black arrow); smear produced directly after blood sampling. Normal appearance of eosinophils, with rounded granules of a pale pink colour. Pappenheim stain, 1000x.

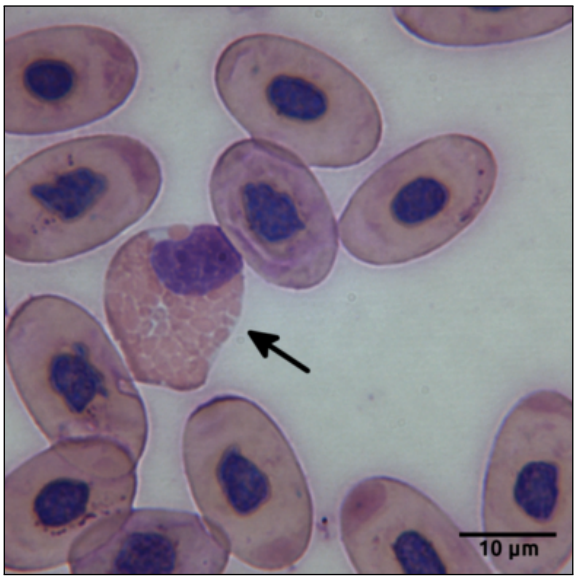

Fig. 4.18. Eosinophil (black arrow); smear produced from blood after storage of 1 h at fridge temperature. Normal appearance. Pappenheim stain, 1000x.

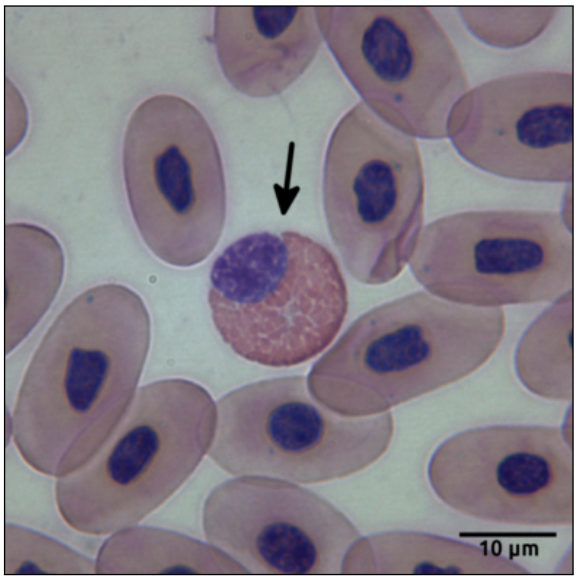

Fig. 4.19. Eosinophil (black arrow); smear produced from blood after storage of 1 h at room temperature. Normal appearance. Pappenheim stain, 1000x.

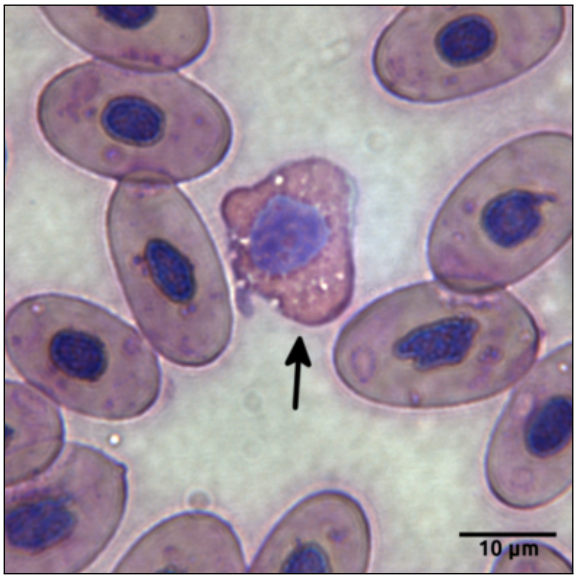

Fig. 4.20. Eosinophil (black arrow); smear produced from blood after storage of 48 h at fridge temperature. Mostly normal appearance, but vacuoles in the cytoplasm appear here. Pappenheim stain, 1000x.

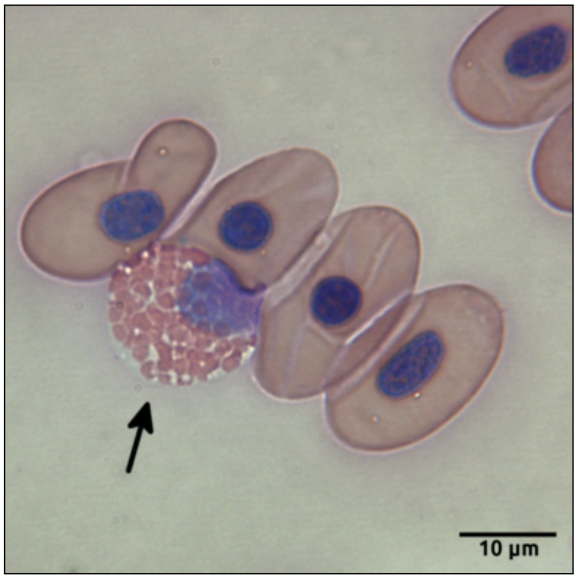

Fig. 4.21. Eosinophil (black arrow); smear produced from blood after storage of 48 h at room temperature. Mostly normal appearance, but the cytoplasmic granules appear less densely packed and small cytoplasmic vacuoles are present. Pappenheim stain, 1000x.

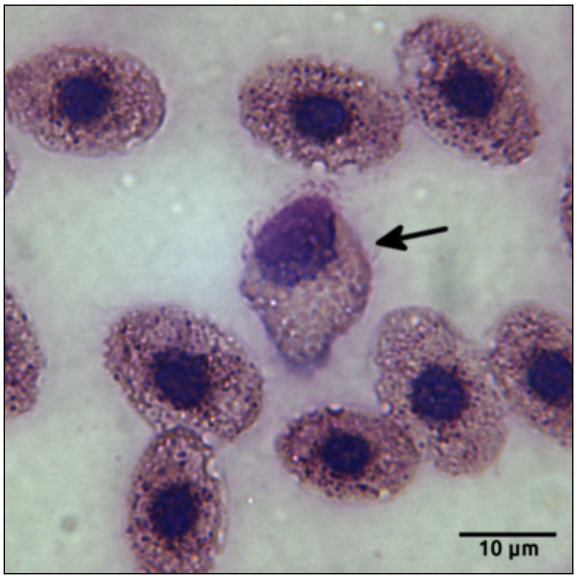

Fig. 4.22. Eosinophil (black arrow); smear produced from blood after storage of 96 h at fridge temperature #1. Cytoplasmic granules are darker than the initial colour and appear smaller. Also note the strong vacuolisation in the cytoplasm of the erythrocytes. Pappenheim stain, 1000x.

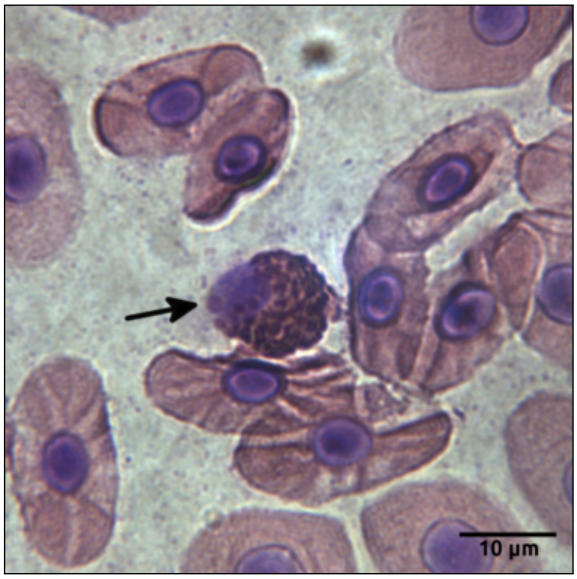

Fig. 4.23. Eosinophil (black arrow); smear produced from blood after storage of 96 h at room temperature #1. Blackberry-like appearance with very dark colour of the cytoplasmic granules. Note the crumpled appearance of the erythrocyte cytoplasm. Pappenheim stain, 1000x.

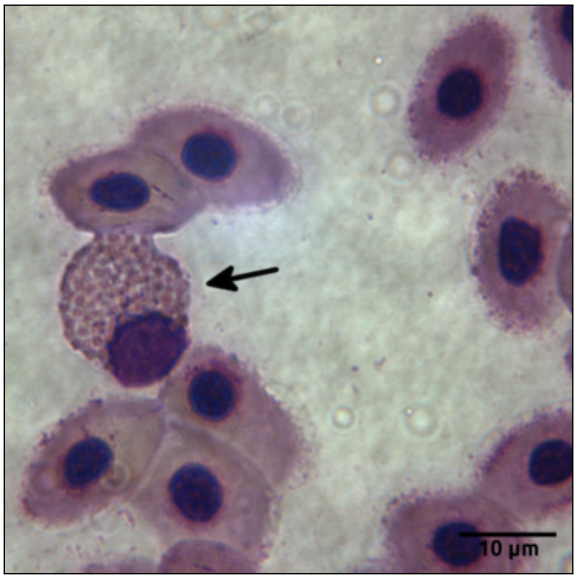

Fig. 4.24. Eosinophil (black arrow); smear produced from blood after storage of 96 h at fridge temperature #2. Cytoplasmic granules are darker and appear smaller. Note the appearance of the erythrocytes with disintegrating margins. Pappenheim stain, 1000x.

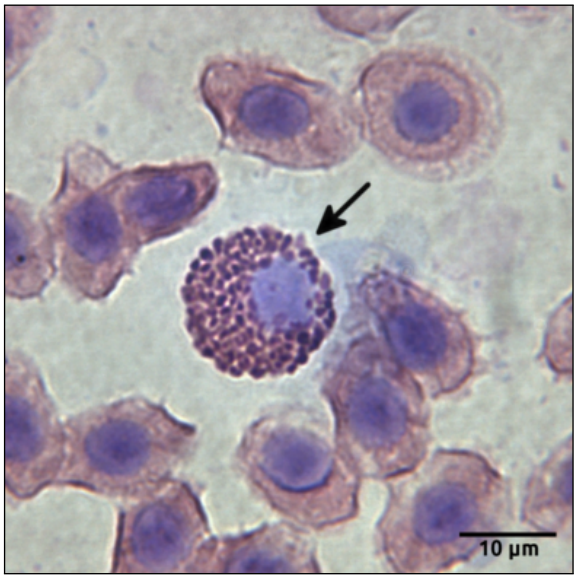

Fig. 4.25. Eosinophil (black arrow); smear produced from blood after storage of 96 h at room temperature #2. Dark cytoplasmic granules that appear smaller than normal. Note the erythrocyte morphology, with loss of oval shape, pale nuclei and disintegrating cytoplasm. Pappenheim stain, 1000x.

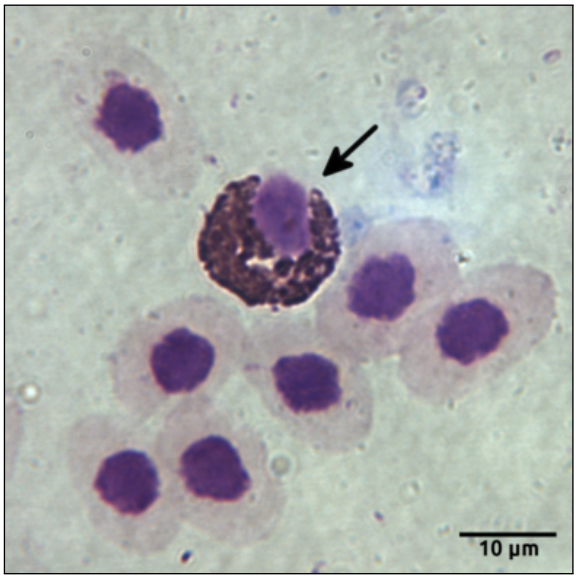

Fig. 4.26. Eosinophil (black arrow); smear produced from blood after storage of 96 h at fridge temperature #3. Very dark colour of the cytoplasmic granules, which are also less demarcated from each other. Note the pale colour of the erythrocyte cytoplasm. Pappenheim stain, 1000x.

4.3.3. Basophils

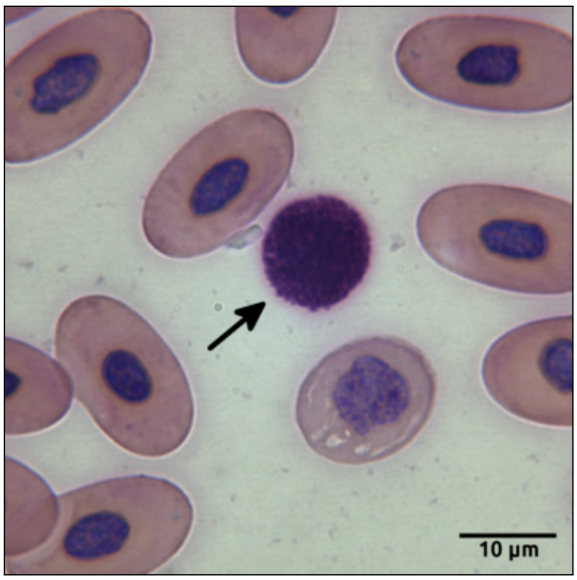

Fig. 4.27. Basophil (black arrow); smear produced directly after blood sampling. Normal appearance. Pappenheim stain, 1000x.

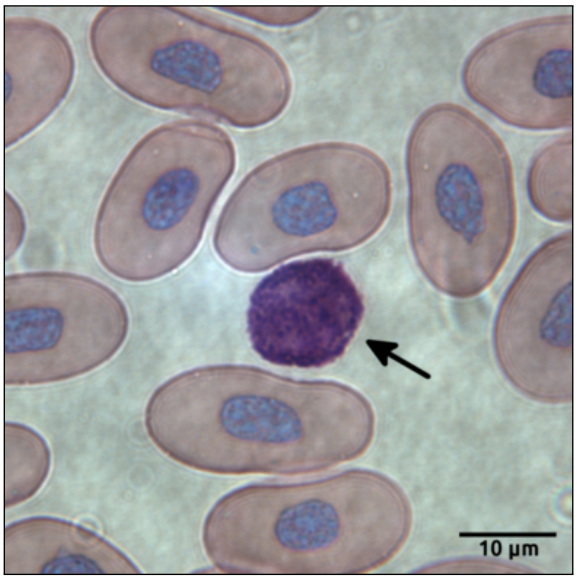

Fig. 4.28. Basophil (black arrow); smear produced from blood after storage of 1 h at fridge temperature. Normal appearance with paler colouring. Pappenheim stain, 1000x.

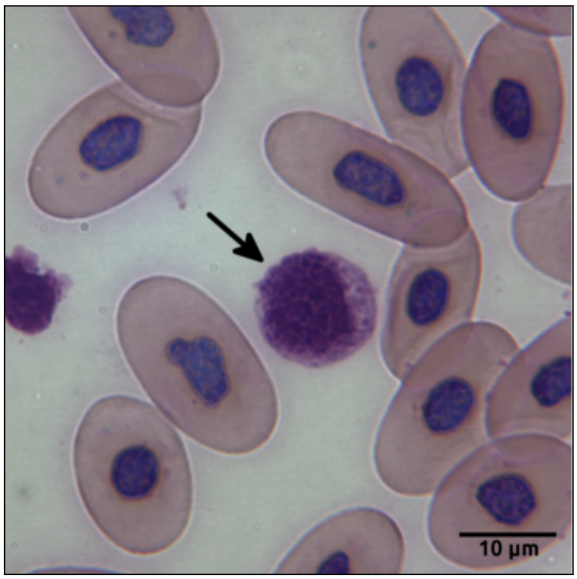

Fig. 4.29. Basophil (black arrow); smear produced from blood after storage of 1 h at room temperature. Normal appearance with beginning degranulation. Pappenheim stain, 1000x.

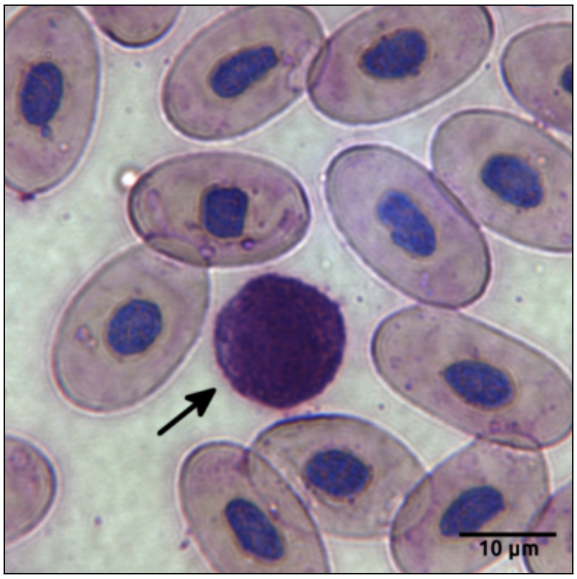

Fig. 4.30. Basophil (black arrow); smear produced from blood after storage of 48 h at fridge temperature. Normal appearance. Pappenheim stain, 1000x.

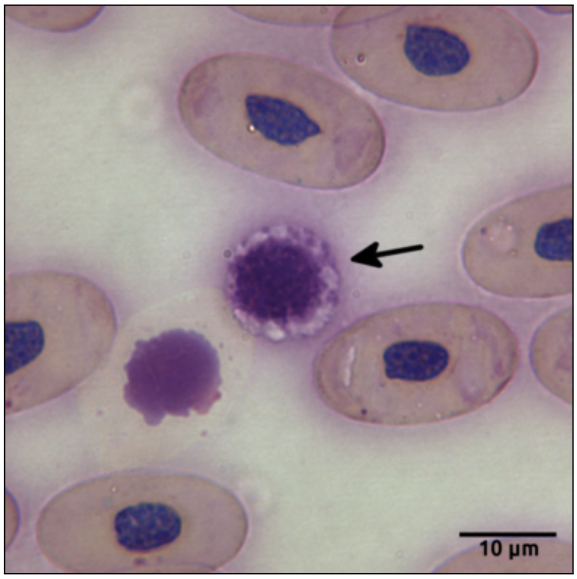

Fig. 4.31. Basophil (black arrow); smear produced from blood after storage of 48 h at room temperature. This basophil appears degranulated, but this could also be a result of the prolonged storage. Pappenheim stain, 1000x.

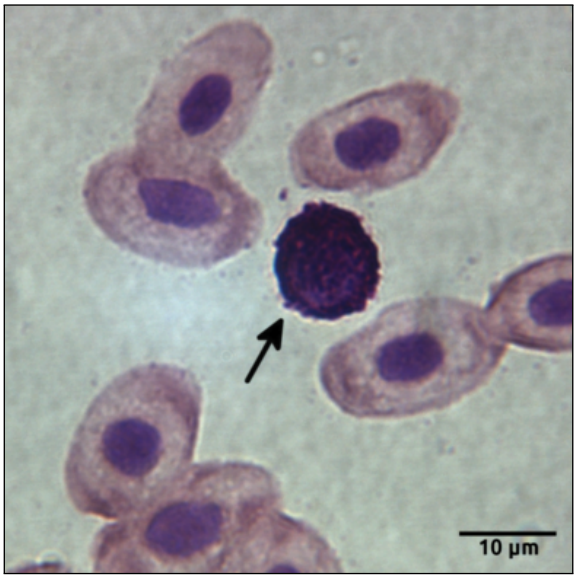

Fig. 4.32. Basophil (black arrow); smear produced from blood after storage of 96 h at fridge temperature. Very dark purple colouring, almost black. Pappenheim stain, 1000x.

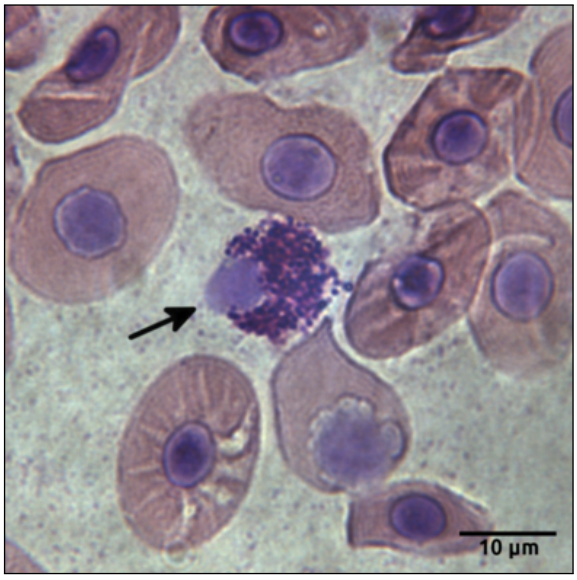

Fig. 4.33. Basophil (black arrow); smear produced from blood after storage of 96 h at room temperature #1. Nucleus unusually visible, with pale colour. Granules do not obscur the nucleus. Note deteriorating erythrocytes with crumpled appearance. Pappenheim stain, 1000x.

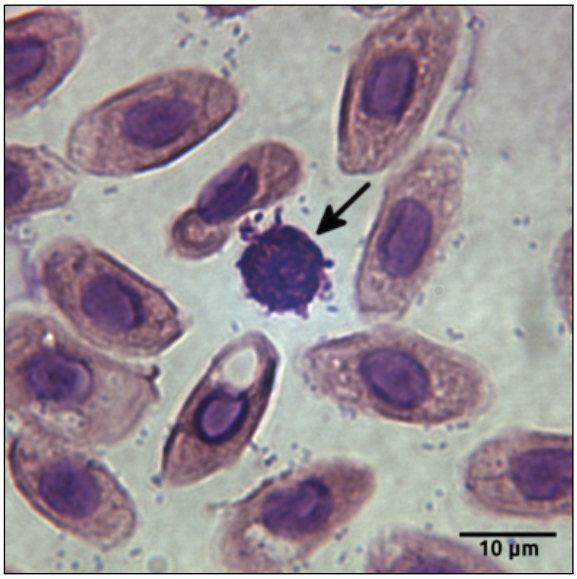

Fig. 4.34. Basophil (black arrow); smear produced from blood after storage of 96 h at room temperature #2. Dark purple basophil, with cytoplasmic and granular appendage. Note deteriorating erythrocytes with cytoplasmic vacuoles. Pappenheim stain, 1000x.

4.3.4. Lymphocytes

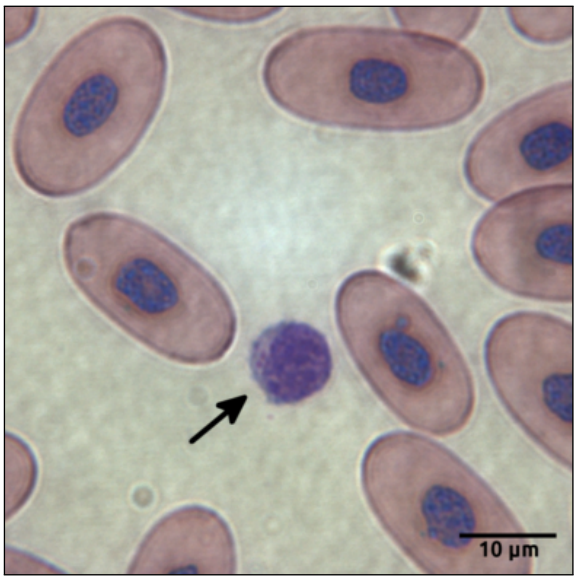

Fig. 4.35. Lymphocyte (black arrow); smear produced directly after blood sampling. Normal appearance of a small lymphocyte. Pappenheim stain, 1000x.

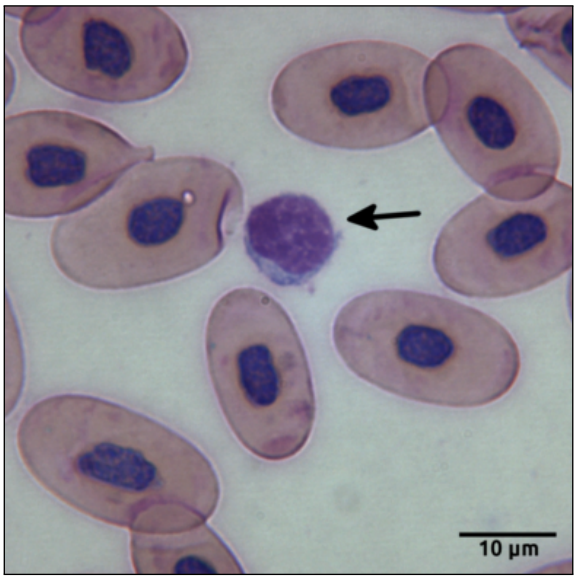

Fig. 4.36. Lymphocyte (black arrow); smear produced from blood after storage of 1 h at fridge temperature. Normal appearance. Pappenheim stain, 1000x.

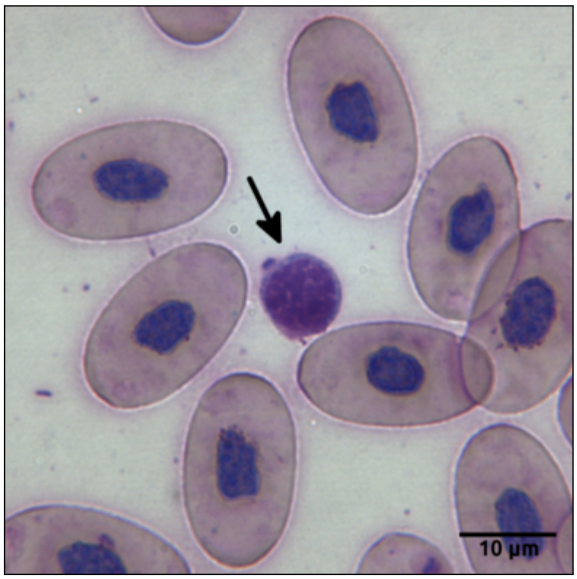

Fig. 4.37. Lymphocyte (black arrow); smear produced from blood after storage of 1 h at room temperature. Normal appearance with very thin cytoplasmic rim. Pappenheim stain, 1000x.

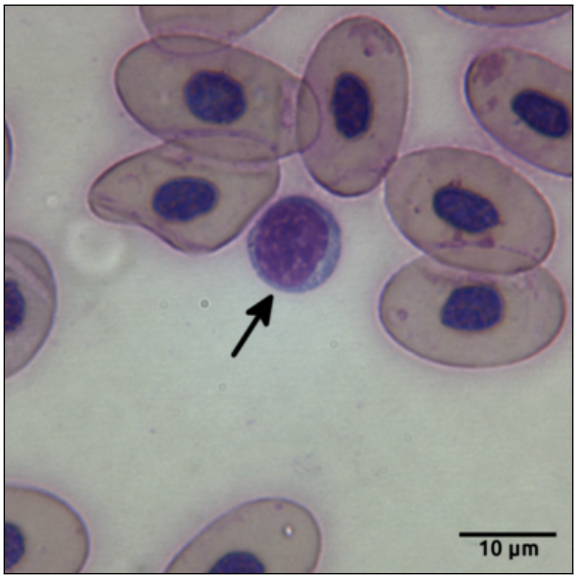

Fig. 4.38. Lymphocyte (black arrow); smear produced from blood after storage of 48 h at fridge temperature. Normal appearance of a larger lymphocyte. Pappenheim stain, 1000x.

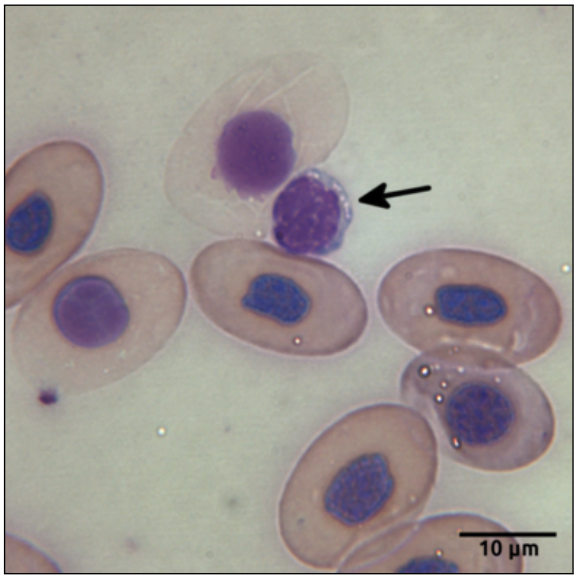

Fig. 4.39. Lymphocyte (black arrow); smear produced from blood after storage of 48 h at room temperature. Normal appearance, with small cytoplasmic vacuoles. Pappenheim stain, 1000x.

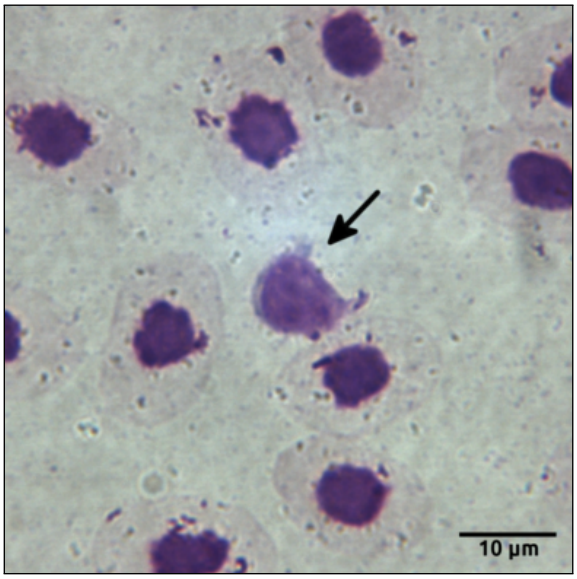

Fig. 4.40. Lymphocyte (black arrow); smear produced from blood after storage of 96 h at fridge temperature. Loss of detail in the chromatin of the nucleus. Note the deterioration of the erythrocytes, with the cytoplasm barely visible. Pappenheim stain, 1000x.

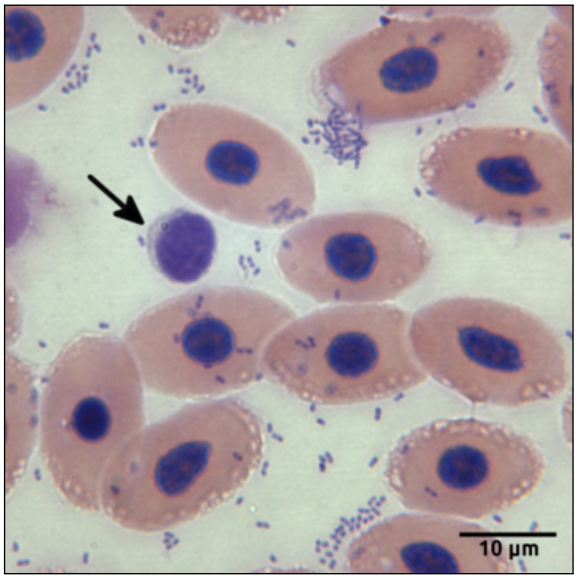

Fig. 4.41. Lymphocyte (black arrow); smear produced from blood after storage of 96 h at room temperature #1. Loss of detail in the nucleus, otherwise relatively normal appearance. Note the bacterial growth and the vacuolisation at the erythrocytic margins. In comparison to Fig. 4.40 and 4.42, these erythrocytes still appear well-preserved. Pappenheim stain, 1000x.

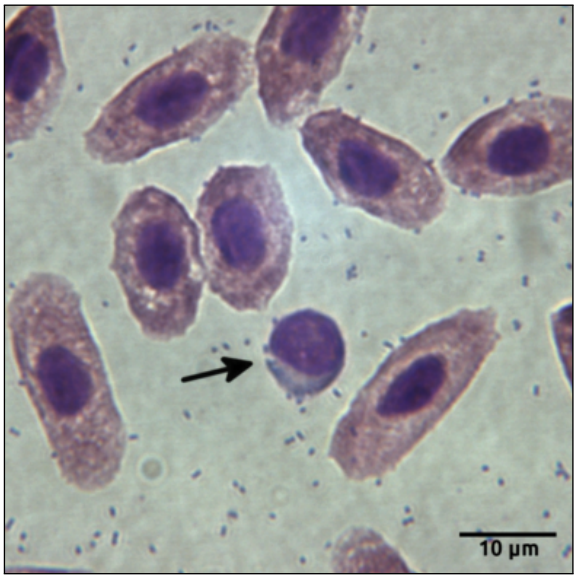

Fig. 4.42. Lymphocyte (black arrow); smear produced from blood after storage of 96 h at room temperature #2. Loss of detail in the nucleus, otherwise relatively normal appearance. Note the deterioration of the erythrocytes and the bacterial growth. Pappenheim stain, 1000x.

4.3.5. Monocytes

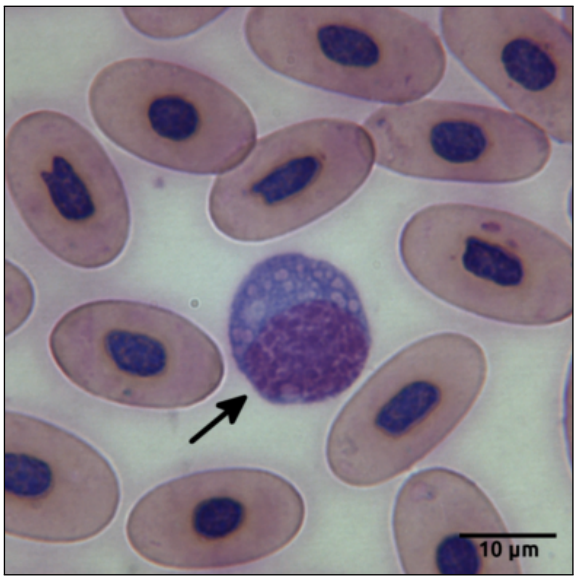

Fig. 4.43. Monocyte (black arrow); smear produced directly after blood sampling. Normal appearance. Pappenheim stain, 1000x.

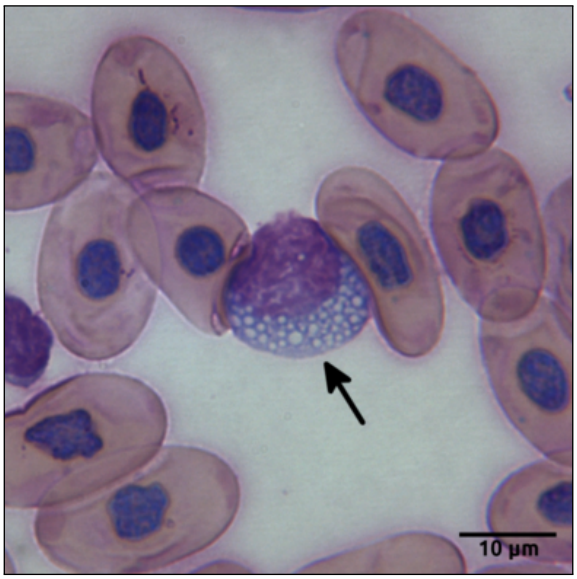

Fig. 4.44. Monocyte (black arrow); smear produced from blood after storage of 1 h at fridge temperature. Normal appearance with small cytoplasmic vacuoles. Pappenheim stain, 1000x.

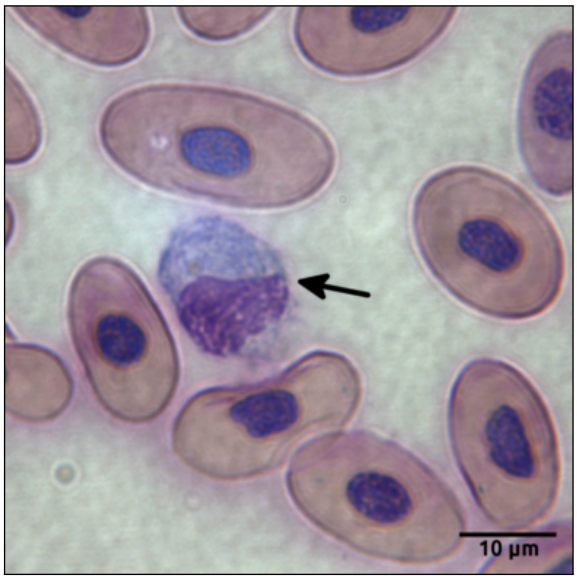

Fig. 4.45. Monocyte (black arrow); smear produced from blood after storage of 1 h at room temperature. Normal appearance, fine granulation visible in the cytoplasm. Pappenheim stain, 1000x.

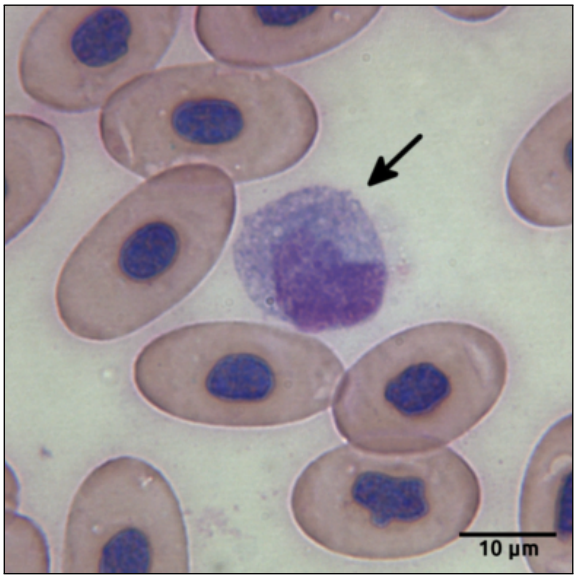

Fig. 4.46. Monocyte (black arrow); smear produced from blood after storage of 48 h at fridge temperature. Normal appearance. Pappenheim stain, 1000x.

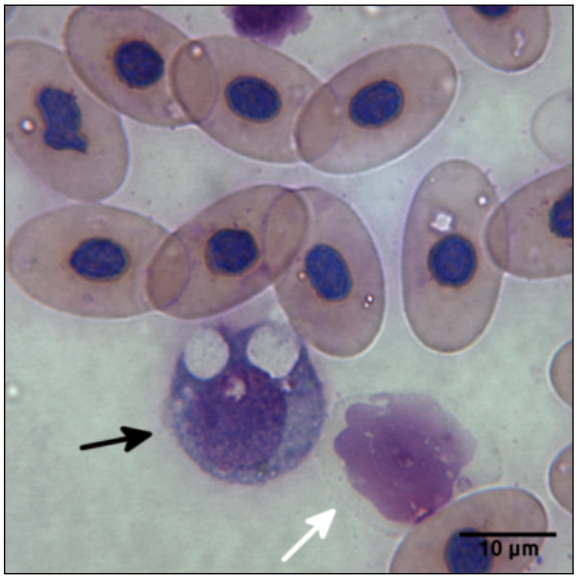

Fig. 4.47. Monocyte (black arrow); smear produced from blood after storage of 48 h at room temperature. Large cytoplasmic vacuoles. Note the lysed erythrocyte (white arrow). Pappenheim stain, 1000x.

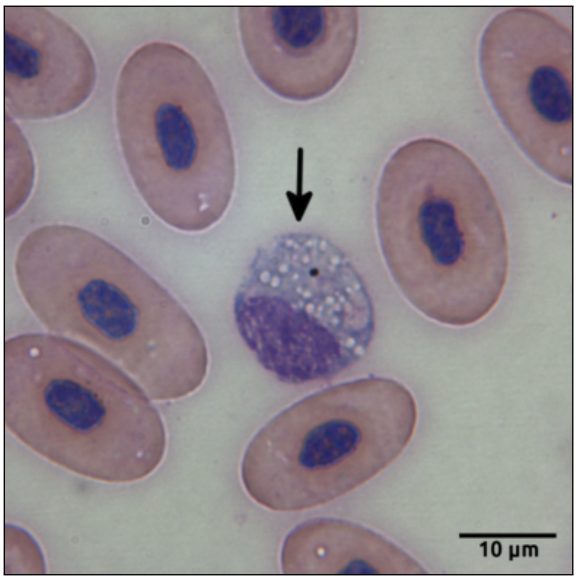

Fig. 4.48. Monocyte (black arrow); smear produced from blood after storage of 96 h at fridge temperature. Normal appearance with small cytoplasmic vacuoles. Pappenheim stain, 1000x.

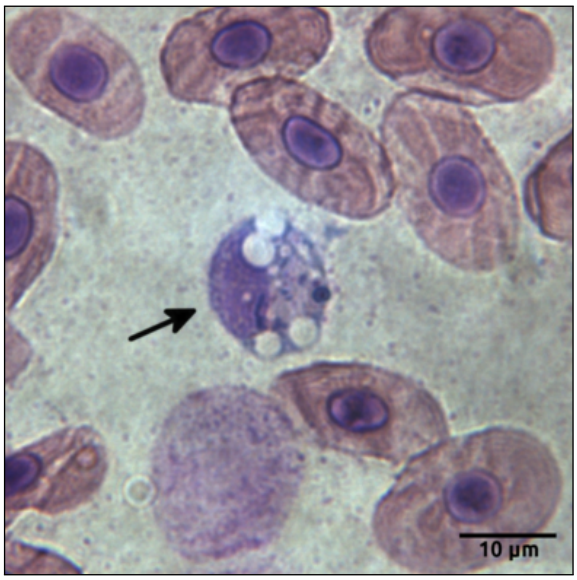

Fig. 4.49. Monocyte (black arrow); smear produced from blood after storage of 96 h at room temperature #1. Large cytoplasmic vacuoles and loss of detail. Note the crumpled appearance of the erythrocytes. Pappenheim stain, 1000x.

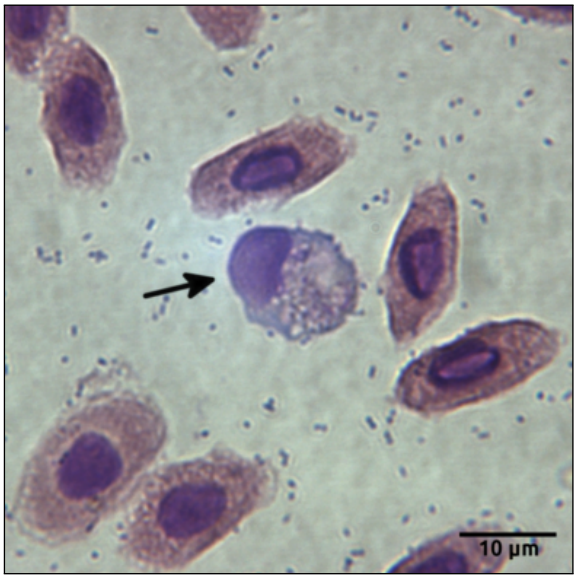

Fig. 4.50. Monocyte (black arrow); smear produced from blood after storage of 96 h at room temperature #2. Small cytoplasmic vacuoles and loss of detail, especially in the nucleus. Note the bacterial growth and deterioration of the erythrocytes. Pappenheim stain, 1000x.

4.3.6. Thrombocytes

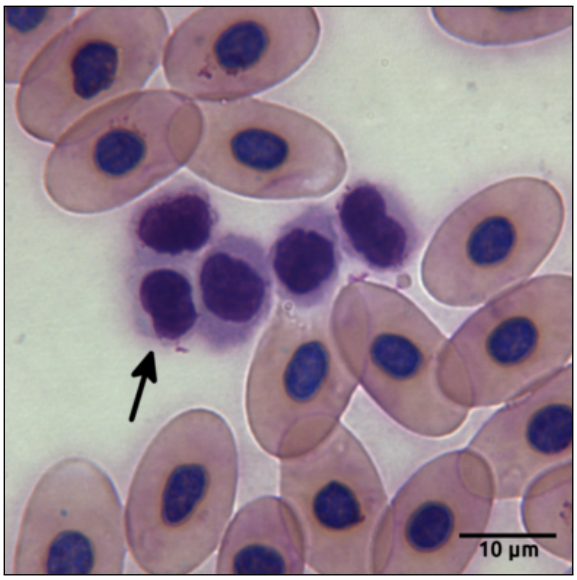

Fig. 4.51. Thrombocytes (black arrow); smear produced directly after blood sampling #1. The more common type of thrombocytes, with irregular shape and small size. Pappenheim stain, 1000x.

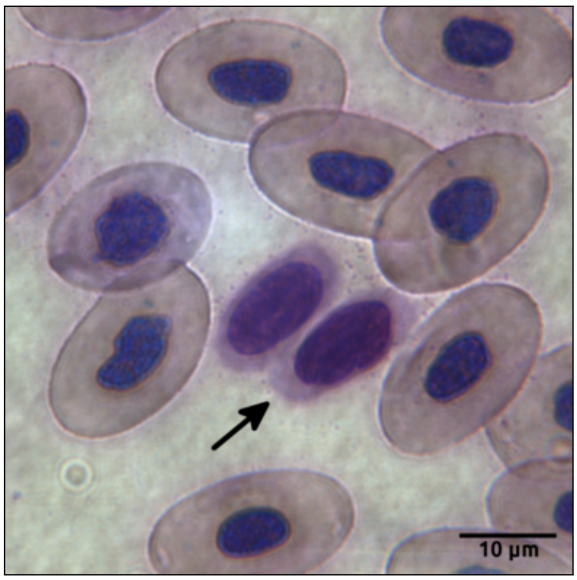

Fig. 4.52. Thrombocytes (black arrow); smear produced directly after blood sampling #2. The less common type of thrombocytes, with regular elliptical shape and larger size compared to the other type. Pappenheim stain, 1000x.

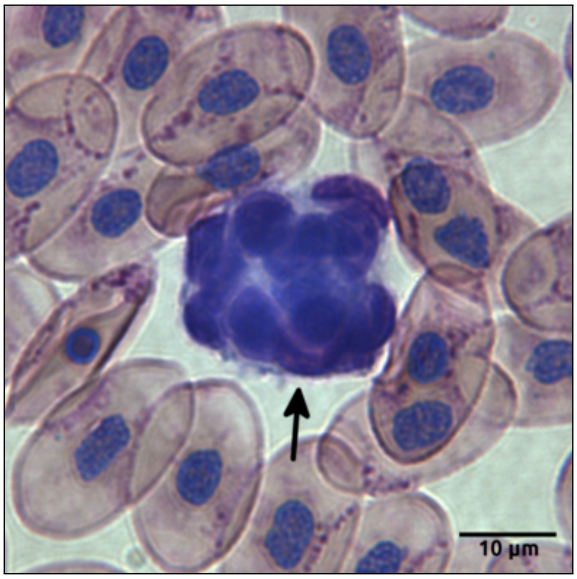

Fig. 4.53. Thrombocytes (black arrow); smear produced from blood after storage of 1 h at fridge temperature. Normal appearance of a thrombocyte cluster. Pappenheim stain, 1000x.

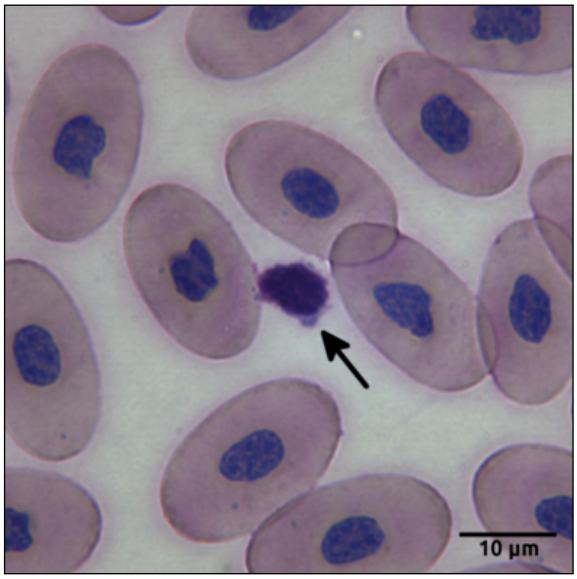

Fig. 4.54. Thrombocyte (black arrow); smear produced from blood after storage of 1 h at room temperature. Normal appearance of a very small thrombocyte. Pappenheim stain, 1000x.

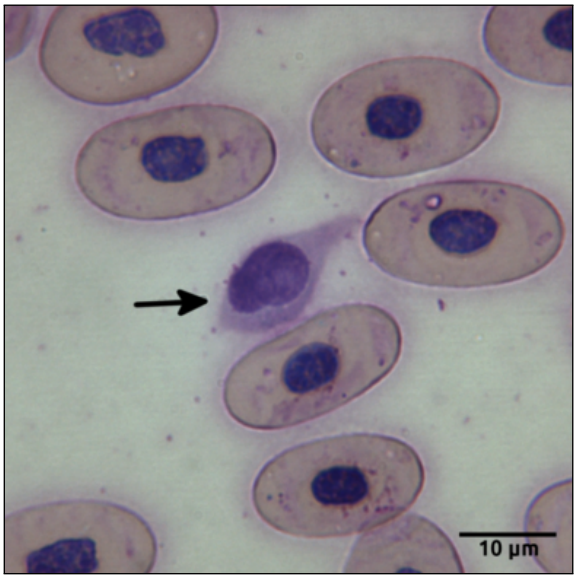

Fig. 4.55. Thrombocyte (black arrow); smear produced from blood after storage of 48 h at fridge temperature. Normal appearance of a spindle-shaped thrombocyte. Pappenheim stain, 1000x.

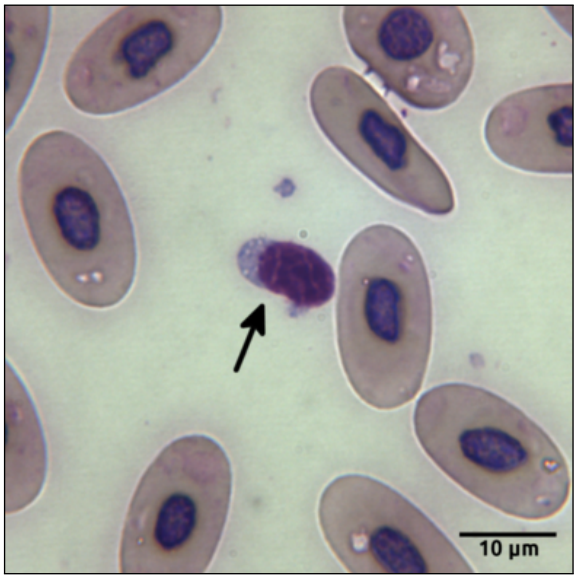

Fig. 4.56. Thrombocyte (black arrow); smear produced from blood after storage of 48 h at room temperature. Small vacuoles in the thrombocytic cytoplasm. Pappenheim stain, 1000x.

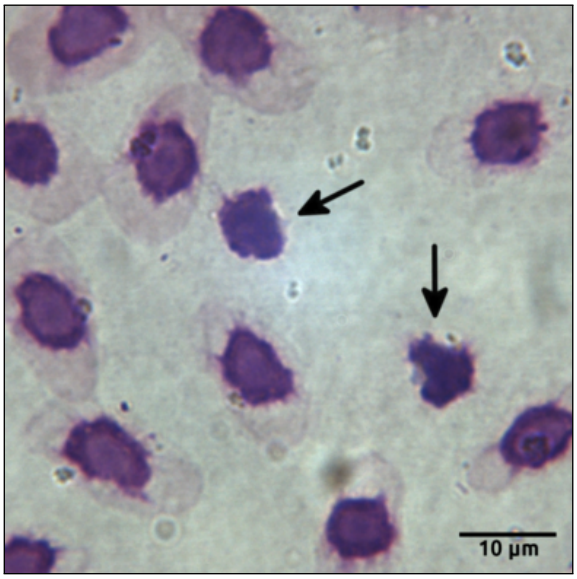

Fig. 4.57. Thrombocytes (black arrows); smear produced from blood after storage of 96 h at fridge temperature #1. Loss of detail. Due to the deterioration of the erythrocytes with almost invisible cytoplasm, in some smears it became difficult to distinguish thrombocytes from erythrocytic nuclei. Pappenheim stain, 1000x.

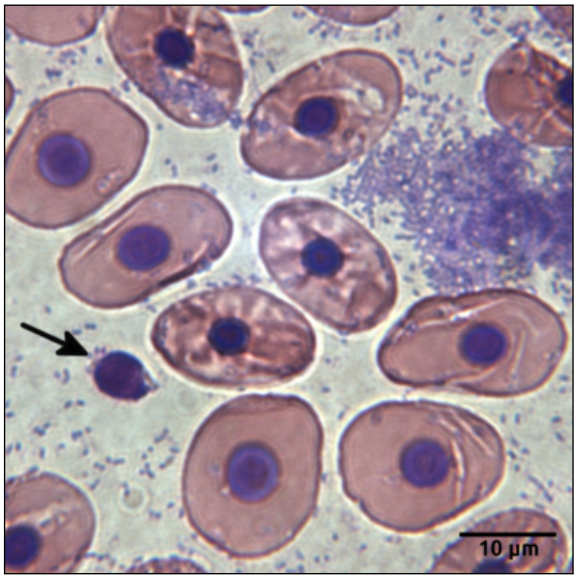

Fig. 4.58. Thrombocyte (black arrow); smear produced from blood after storage of 96 h at room temperature #1. Also note the heavy bacterial growth and deterioration of the erythrocytes with a crumpled appearance. Pappenheim stain, 1000x.

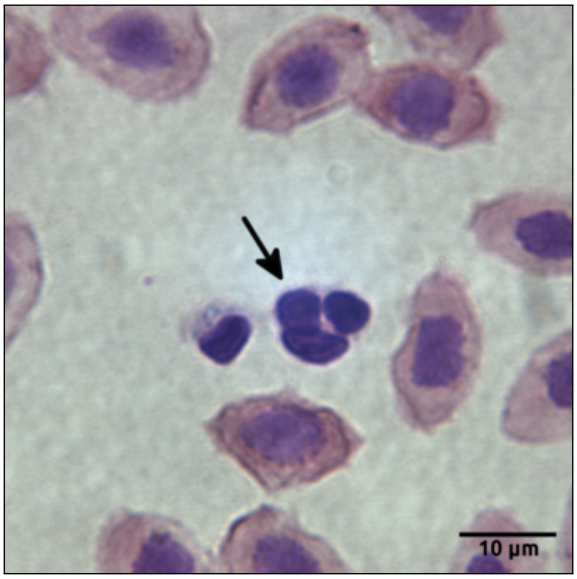

Fig. 4.59. Thrombocytes (black arrow); smear produced from blood after storage of 96 h at fridge temperature #2. Thrombocytes appear here as small spots with a dark purple colour, cytoplasm is barely present. Pappenheim stain, 1000x.

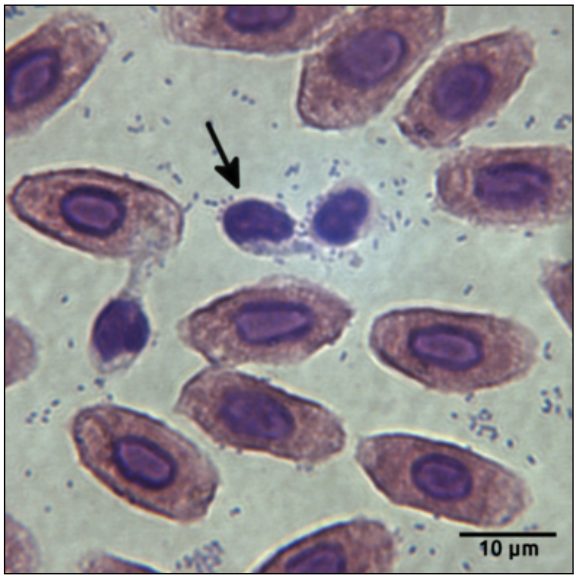

Fig. 4.60. Thrombocytes (black arrow); smear produced from blood after storage of 96 h at room temperature #2. Also note the bacterial growth and deterioration of the erythrocytes with a disintegrating appearance. Pappenheim stain, 1000x.

5. Discussion

Changes in different blood values related to different storage conditions are not completely unknown in other species than Hermann's tortoises. The blood of macaws has been found to develop a strong haemolysis within 12 h of storage, regardless of the investigated anticoagulants (sodium citrate, EDTA, lithium heparin). When comparing the haemolysis in macaw blood samples with samples from burmese pythons, the latter were more stable up to 24 h [Harr et al., 2005]. Variations in the haematocrit, haemoglobin concentration, red blood cell count and white blood cell count were found after storing blood from cattle, pigs and goats at room temperature respectively fridge temperature (for details, see chapter 2.2.2: Influence of the handling of blood specimens on results) [Ihedioha and Onwubuche, 2007]. In canine blood samples, changes were found for the mean corpuscular volume and the white blood cell counts as well as the platelet counts after storage for up to 48 h at room temperature (for details, see chapter 2.2.2: Influence of the handling of blood specimens on results) [Médaille et al., 2006]. Obviously there are influences of the storage conditions on miscellaneous blood values in different species. The ASVCP has published Quality Assurance Guidelines for the preanalytical handling of blood samples, but in the case of reptilian blood samples these remain rather vague and are largely unknown [ASVCP, 2009].

Reptilian and avian blood cells differ from mammalian blood cells in some aspects. The erythrocytes and thrombocytes of reptiles and birds are nucleated while those of mammals show no nucleus. While reptilian erythrocytes mostly have nuclei with a slightly erratic margin, those of birds are regular and smooth [Campbell and Ellis, 2007]. In mammals the leucocytes are differentiated into the basophilic, eosinophilic and neutrophilic granulocytes as well as monocytes, lymphocytes and mast cells. The

nomenclature of reptilian leucocytes is not completely consistent, but mostly they are separated into heterophils, basophils and eosinophils as well as monocytes, lymphocytes and plasma cells, with the existence of the latter being controversial (see chapter 2.3.3: Leucocytes). While these differences are well-known, it must be taken into account that physiological differences may not only be restricted to animal classes but great variations can also occur between various species within the same class.

In the presented study the influence of the storage time and storage temperature of blood samples from Hermann's tortoises *(T. hermanni)* on the quality of the blood smear and the differential blood count was investigated. The quality of the blood smear was examined by evaluating previously defined criteria: The macroscopic and microscopic quality, the bacterial growth and the cytoplasmic vacuoles in the erythrocytes. In addition, a differential blood count was established. While the macroscopic quality did not show a strong influence of storage time and temperature, the microscopic quality mostly declined with prolonged storage in both temperatures. The bacterial growth and the cytoplasmic vacuoles in erythrocytes both increased distinctly with prolonged storage at room temperature. For the fridge temperature, the increase was less distinct, but existent. The leucocyte percentage values showed a clear difference between the two storage temperatures. While the percentage distribution remained uninfluenced by fridge temperature, the specimens stored at room temperature yielded a decline of cells identified as heterophils and an increase of lymphocytes with prolonged storage. Additionally, the morphological changes in the blood cells were documented with microscopic photos. These changes did not develop completely uniform in all slides during storage, but the most relevant and frequently occurring changes were documented exemplarily, such as changes in cell size and colour as well as lysed cells and cytoplasmic vacuoles. In some cases it became difficult to distinguish between heterophils and eosinophils. The differentiation of thrombocytes and small lymphocytes was difficult to start with and this problem increased with prolonged storage.

5.1. Method discussion

5.1.1. Animals

It was decided to use Hermann's tortoises for this study, because these tortoises are one of the most commonly kept reptile species in Germany. From this concludes that they are one of the most frequently presented reptile species in veterinary surgery. Among the 456 chelonians presented at the Klinik für Vögel, Reptilien, Amphibien und Zierfische of the LMU Munich within one year (July 2014-June 2015) were 275 Hermann's tortoises, which equates to 60,3 %.

The blood samples used in this study were obtained in the context of the propaedeutics classes for students of veterinary medicine at the Klinik für Vögel, Reptilien, Amphibien und Zierfische of the LMU Munich. For this study, the required amount of blood was taken out of the samples before they were discarded. The survey was restricted to male animals, as they usually have a longer tail than females, allowing easier access of the Vena coccygealis dorsalis for blood collection. Blood specimens of 0,33 ml were necessary and a minimum body weight of at least 200 g was assigned to absolutely ensure that the blood volume was sufficient for blood sampling without harming the animal, also bearing in mind the repeated samplings after one month. The actual body weights of the tortoises ranged from 445-1085 g, so that the smallest animal had the double minimum weight.

Since the emphasis of this study was on how the storage time and temperature influence the results, as opposed to the indiviual animals and other influences, it was attempted to standardise the procedure as much as possible for a better comparability. To achieve this, it was considered necessary to use animals of the same sex and to use the same individuals repeatedly, as this would rule out a variation due to different individuals.

In veterinary practice it is recommended to tortoise owners to have a blood test run at least once every year in line of a routine health check before the tortoises are hibernated to review their health status, as sick animals should not be hibernated. This not only allows

the veterinarian to establish individual reference intervals over time, which facilitates the early recognition of diseases in the individual animal. Repeated blood examinations are also necessary for the veterinarian to monitor the success of therapy in a sick animal, as has been recommended by Campbell [2006], who underlined the utility of repeated blood examinations in a single animal. In this study an interval of at least three weeks in between the blood samplings was set, which was considered a safe time for blood renewal and recovery from overall strain and also allowed the study to be carried out within one summer activity period of the tortoises.

Since no signs of illnesses were found in the clinical examinations and the X-rays, the animals used were regarded as healthy despite the lowered LDH values, as this was not considered to require treatment.

5.1.2. Evaluation of blood smears

The evaluation of all blood smears was conducted by the same person and comprised of several steps. First, the macroscopic quality was rated. Subsequently, the microscopic evaluation followed, beginning with a differential blood count of 100 leucocytes. During the performance of the differential blood count, the microscopic quality, the bacterial growth and the cytoplasmic vacuoles in the erythrocytes were also observed and documented with graded assessments.

It was decided to evaluate the smears by a single person taking advantage of the fact that then the same criteria were used consistently. A problem using this strategy can arise because of a certain subjective bias of the evaluating person, which is difficult to rule out completely. In the presented study it was tried to restrict this bias by using predefined criteria for the macroscopic and microscopic quality and a staged assessment for the amount of bacterial growth and the cytoplasmic vacuoles in erythrocytes. Nonetheless, criteria evaluated by humans always show a more or less distinct bias due to slightly different assessments of the same information by different persons. To integrate this bias adequately during the clinical assessment of a differential blood count result, it would

be necessary to have further studies comparing leucocyte differential counts obtained by several persons with varying stages of experience. This would allow to assess the influence of the examiner's experience on the reliability of the results.

Corrons et al. [2004] listed vacuoles in human blood cells as a quality criteria and during preliminary tests it became apparent that especially the erythrocytes would begin to show cytoplasmic vacuoles when the blood sample was stored for longer periods. The preliminary assessments also showed an increasing bacterial growth in the longer-stored samples, therefore these two criteria were included in the final evaluations.

5.1.3. Statistical evaluations

When examining the obtained leucocyte values, it became apparent that the basophil, monocyte and plasma cell values represented only a small percentage of the differential blood count and were not influenced strongly by the various storage conditions. Therefore it was decided to pool these data as the group "rest" for the statistical evaluation of the leucocyte values. This group "rest" was weighted with 1, while the heterophil, eosinophil and lymphocyte values were weighted with 2 to emphasise the differences resulting from the storage conditions. The different weighting of the cell types was established for a better visualisation of the relevant changes in distribution within the differential blood count. Hereby the cell types with the largest percentages were considered more representative for the influence of storage conditions of blood samples on the differential blood count. Basophils, monocytes and plasma cells appeared in much lower percentages (basophils represented ≤ 5 % in 97.14 % of the smears, monocytes ≤ 5 % in 99.04 % of the smears). These numbers were considered too low to allow for a representative comparison of an influence of storage conditions on the percentage of these cells.

5.2. Discussion of results

During this study, two types of thrombocytes were found and described for the first time in *T. hermanni*, one with more irregular shapes and a darker colouring as well as one with regular, elliptical shape and a lighter colouring (Fig. 4.51 and 4.52). The possibility that two types of thrombocytes might also exist in other tortoise species is supported by a study of Knotková et al. [2002], who reported two types of thrombocytes in the russian tortoise (*A. horsfieldii*) and described these as oval with transparent cytoplasm and rectangular with lightly basophilic cytoplasm, respectively.

The effects of prolonged storage of blood might impair the evaluation of blood smears and therefore the consequences drawn for tortoise patients have clinical relevance. With prolonged storage, some of the leucocytes became easier to confuse with other cell types, especially heterophils with eosinophils and lymphocytes with thrombocytes. Together with the decreasing microscopic quality of the blood smears this increases the risk of misinterpretation and therefore incorrect results. Also, the distinctly longer time needed for a complete evaluation of a blood smear with poor overall quality should not be disregarded. Occasionally the poor quality could even lead to the blood smear not being fully evaluable at all, because it became impossible to find 100 leucocytes for differentiation. This has been the case in three of the blood smears in this study, all made from the same blood sample stored for 96 h at room temperature before the smears were produced.

In this study, it became obvious that experience in evaluating tortoise blood smears is especially important in blood samples with prolonged storage time. At the beginning of the evaluations, the counts of cells in the smears identified as thrombocytes were relatively low and stabilised itself at the evaluation of about the 35th smear of the total number of 210. During re-evaluation of the first 37 smears it became clear that in the first evaluated smears not all thrombocytes were correctly detected leading to the fact that thrombocyte countings were too low. This effect, however, was restricted to the thrombocytes, the leucocyte counts did not show this problem. This is most likely explained by increasing experience of the author during the progressing investigation

leading to a more confident identification of thrombocytes. As the lymphocyte counts were unaffected, the differentiation of small lymphocytes and thrombocytes was not the source of the problem. This was unexpected, since the trouble in differentiating small lymphocytes and thrombocytes in reptiles is well-known [Frye, 1991; Heard et al., 2004]. Nonetheless, the importance of training and experience of the evaluating person must not be underestimated.

The author had gained experience in evaluating blood smears during a year's occupation at the clinical laboratory of the Klinik für Vögel, Reptilien, Amphibien und Zierfische of the LMU Munich. The preliminary assessments conducted before this study provided further practical training. As mentioned above, to assess the influence of the examiner's experience on the results, studies with several evaluating persons with various stages of experience could be performed.

5.2.1. Macroscopic quality

The macroscopic quality showed no apparent influence of the storage time or the storage temperature. The wide distribution of quality values found in all temperature and time storage combinations points towards influences outside of the storage time and temperature. A certain irregularity in the smear production, that means, external human influence, is likely, as it is impossible for a person to always maintain the exact same angle and speed of the top slide during the production of blood smears. However, other effects, for example the storage conditions, individual tortoises or the season, cannot be excluded, especially if the viscosity of the blood is affected.

5.2.2. Microscopic quality

The microscopic quality was reduced clearly with prolonged storage, with this effect being most distinct in the specimens stored at room temperature. This result confirms clinical and empirical experience, as continued storage in both temperatures often led to more

colour precipitates and spotting in the smear. The decline in microscopic quality could also be assigned to more agglutinations of blood cells with continued storage. These larger cell aggregates would then be pushed towards the end of the smear. This would impair the even distribution of cells, which was included in the evaluation of the microscopic quality.

Surprisingly, a low percentage of smears showed a slight improvement in the microscopic quality after storage compared to smears produced immediately after venipuncture. This phenomenon might merely reflect a random distribution around a median value, but could also be explained by a certain bias during the evaluations, which must be accepted when the same person conducts all the evaluations, since it is not viable to rule out this bias completely. While care was taken to avoid lymphodilution during the blood sampling, nonetheless it is possible that some specimens were contaminated with lymph. Lymphodilution is a known problem in reptile haematology, but this problem has not been investigated experimentally as the possible cause of poor monolayer formation in reptilian blood samples, as discussed frequently among specialists in exotic animal medicine. The even distribution of the cells was included in the evaluation of the microscopic quality and therefore the default values could be affected by this problem. Also, it is not known to the author how lymph reacts to prolonged storage and it may be possible that effects of the lymph fraction actually diminish during storage, which could explain the slight improvements in the microscopic quality in some smears. Further investigations are necessary to clarify the effects of lymph contamination on the storage properties of reptilian blood.

5.2.3. Bacterial growth

The bacterial growth was unchanged after 1 h of storage in both storage temperatures and increased with prolonged storage. The differences became most obvious in the samples stored at room temperature, where 90 % of the smears showed a decline after 96 h compared to only 10 % of the smears from the samples stored at fridge temperature for 96 h. The bacterial growth in a blood smear can interfere with the evaluation, especially

when clumps of bacteria begin to obscure cells, which can lead to confusion about the identification of cells and also lengthens the time needed for the evaluation. Bacteria overlay in the cytoplasm of blood cells could also be mistaken for pathologic signs by less experienced examiners. The development of the bacterial growth over time showed that storage of blood samples at fridge temperature is essential for an unhampered evaluation of blood smears. This is especially the case if blood specimens cannot be evaluated directly, but for various reasons have to be stored for up to 96 h or even more before producing the smears.

While bacterial growth occurred in all blood samples stored at room temperature for 96 h, it was not present in all smears from aliquots of these samples. 10 % of these smears remained completely without bacterial growth. The bacterial growth can most probably be assigned to a not completely sterile blood sampling, for example contamination material in small skin folds on the tail of the tortoise despite cleaning and disinfection or, unlikely, because the blood collection tubes were not completely sterile on the inside but were contaminated during the transfer of the blood. There is also the possibility of a latent bacteriaemia in the tortoises which could not be discovered during the health checks. An even longer storage of the blood samples would most likely have lead to a further increased amount of bacterial growth, because small initial contaminations would have had more time to proliferate. The storage at fridge temperature on the other hand prohibits an increase in the bacterial growth and therefore even heavy initial contamination would not affect the results.

5.2.4. Cytoplasmic vacuoles in erythrocytes

The cytoplasmic vacuoles in erythrocytes increased with storage of 48 h and 96 h in both temperatures. The increase was stronger in the smears from samples stored at room temperature, where only 3.34 % of the smears remained unchanged after 96 h compared to 30 % in the fridge temperature specimens.

Cytoplasmic vacuoles in blood cells are a known artefact in blood samples stored for too long at room temperature. This artefact is mentioned in human neutrophils and monocytes after a storage of three hours at room temperature, for example [Corrons et al., 2004]. It is not known to the author how exactly these vacuoles are induced in erythrocytes and other blood cells during prolonged storage, especially because they did not occur in all blood smears produced from blood after prolonged storage. Like the bacterial growth, cytoplasmic vacuoles can be problematic during the evaluation. Less experienced examiners could confuse the storage artefact with a sign of disease, for example intracellular haemoparasites such as *Sauroplasma* or merozoites of *Plasmodium*, which were reported to appear as vacuoles in the erythrocytic cytoplasm [Halla et al., 2014; Halla, 2015]. Therefore, care should be taken to avoid the formation of this artefact by producing the blood smears within 48 h after the sampling, when stored at fridge temperature. In this study, the samples stored at fridge temperature showed a distinct increase in the cytoplasmic vacuoles in erythrocytes after 96 h storage time, but not at 48 h where the vacuoles were only increased slightly in just 3.34 % of the smears. For the room temperature, further studies are required to narrow down the storage time when cytoplasmic vacuoles in erythrocytes begin to occur. In this study, after 48 h, more than 50 % already showed a decline in this attribute, but 100 % of the smears were still free of vacuoles after storage for 1 h.

5.2.5. Influence of the single animal and the month of blood sampling on the quality indices

In the present study, not only the storage conditions of the blood samples before producing the smears, but also the individual animal influenced the distribution of the quality indices as well as the month of blood sampling. This is correspondent to the current state of knowledge about the influence of seasons on reptilian blood values [Lawrence and Hawkey, 1986; Lawrence, 1987; Christopher et al., 1999; Chung et al., 2009; Scope et al., 2013]. The presented study shows for the first time that season and individual animal do not only influence the blood parameters themselves but also have effects on the quality indices determined here. This means that the storage conditions were not the only

factors influencing the distribution of the quality indices. Nevertheless, the results of this evaluation reinforce the current state of knowledge not to disregard a seasonal influence and a great range of variation inbetween individual animals when evaluating reptilian blood samples.

5.2.6. Distribution of leucocyte values

In comparison with the reference values found in literature sources, the obtained differential blood counts corresponded most closely to those published by Lawrence and Hawkey [1986] for *T. hermanni* and *T. graeca* (Table 2.1). The ranges given by Kölle [2014] also suited the obtained values, but these were not specific for tortoises.

The distribution of the leucocyte percentage values remained mainly constant for the fridge temperature specimens, while the room temperature specimens showed a decline in heterophil counts after storage for 48 h which continued distinctly until 5 % heterophils were found after 96 h compared to 29 % in the smears produced immediately after venipuncture. The lymphocyte count was increased accordingly after 96 h to 78 % as opposed to 58 % in the direct smears. The eosinophil count remained mainly the same throughout the storage times, with a slight increase to 19 % after 48 h, while the other times showed a range from 10-14 %.

Although the heterophils were often easy to confuse with eosinophils in the smears from samples stored for 96 h at room temperature, the eosinophil percentage count did not increase in the same way the heterophil count declined. Therefore, this difficulty in differentiating cannot be the reason for the higher heterophil counts. In many of these smears, heterophils were not found at all or only in very low numbers (0-5 %). This phenomenon resulted in a longer time needed to find 100 leucocytes for the completion of the differential blood count and this additional time allowed for the finding of more lymphocytes, which were increased to 78 % from 58 % in the direct smears. Since heterophils and lymphocytes are unlikely to be confused with each other and eosinophils were difficult, but not impossible to differentiate, it is not likely that the heterophils were

falsely accredited to another leucocyte group. Additionally, the values of eosinophils and the group "rest" (comprised of basophils, monocytes and plasma cells) were not strongly influenced by the storage conditions. The heterophils are probably the leucocytes in *T. hermanni* that are less stable than the other leucocytes and therefore begin to deteriorate first. This would lead to the lack of heterophils found in this study in smears from samples stored at room temperature for 96 h. To avoid such a strong falsification of the obtained results it is necessary to store the blood samples in the fridge if they cannot be attended to immediately.

5.2.7. Morphological changes in blood cells

During the study, various morphological changes in blood cells were found with continued storage. Most remarkable in all cell types was a certain loss of detail in the finer cell structures, such as the chromatin clumping inside the nuclei. This loss of detail is probably explained best by a deterioration of the cellular structures, which would then show up less distinct in the stained smear. Also, the biochemical cellular composition could change with prolonged storage due to metabolic processes which might lead to a change in the absorption of the stain, this would also make the cells appear differently than the initial appearance. The decline in detail may also be a result of the prolonged exposure of the blood cells to the anticoagulant in the blood collection tube, in this case lithium heparin. Similar findings have been described in avian blood, where [Walberg, 2001] reported blood to stain less well after being exposed to heparin for several hours and described the nuclei as smudged and the granules as staining very faintly, but he did not specify the stain used.

The overall decline in the appearance of the blood cells observed with prolonged storage lead to difficulties in the differentiation of some cell types, for example heterophils became easier to confuse with eosinophils and small lymphocytes were even more difficult to distinguish from thrombocytes than under circumstances of short time intervals between venipuncture and production of the smears.

5.3. Aims and limitations

One of the aims of the presented study was to compare the interpretability in blood smears from *T. hermanni*, after storage of the blood samples at different temperatures for varying durations. It was decided to evaluate the differential blood count for comparisons, because this was deemed of relatively high importance in the evaluation of a dried blood smear and could be ascertained from fixed and sealed blood smears. For the practical realisation it was important to produce blood smears that could be stored indefinitely in a fixed and sealed state. On the one hand this was necessary for the opportunity of preliminary assessments without influencing final results and on the other hand it allowed for smears being stored for later evaluation. The possibility of a later evaluation was necessary for the comparison of the condition of samples at the same point of time.

The comparatively small number of four tortoises was chosen partly due to limitations in the elaborate captive husbandry of these animals. Part of the choice was also to reduce the overall strain on experimental animals by using less animals but sampling these more often rather than using more animals. However, the main reason to limit the number of animals was to standardise the blood material as much as possible by reducing a potential effect of individual animals on the quality parameters in order to detect an influence of storage conditions.

A determination of reference values of clinically relevant parameters was not the aim of this study, therefore the parameters and evaluation methods have not been chosen purely from a clinical point of view. The emphasis was placed upon using parameters appropriate as quality parameters for comparison of the different storage conditions. A different approach using different measurement parameters would certainly have been possible, but might have lead to the same results. While it would have been interesting to have another person with more or less experience in evaluating blood smears re-evaluate the smears for comparison, this was not possible due to practicability reasons. Further limitations of the study might be seen in the semiquantitative ratings of the bacterial growth and the cytoplasmic vacuoles in erythrocytes, which were used because of practicability reasons.

More exact quantitative ratings might have lead to more exact results. The same goes for the semiquantitative rating of the morphological changes in blood cells.

5.4. Conclusions and recommendations

As there have been no studies about morphological changes in chelonian blood cells with prolonged storage at different temperatures before, the hereby presented investigation provides a reference of possible changes and a guideline of handling recommendations for practitioners, so as not to mistake storage artefacts for pathological signs. In addition, the normal appearance of leucocytes and thrombocytes in *T. hermanni* has also been documented to provide an assistance in the identification of blood cells from *T. hermanni* for less experienced examiners.

In veterinary practice there are mainly two situations imaginable where samples are stored for longer periods of time before producing and evaluating smears. Firstly, the storage over a weekend, when there is no staff available for the job and secondly, the shipping of samples to an external laboratory for evaluation. This study investigated the influence of the different storage temperatures after 1 h, 48 h and 96 h to simulate situations such as these and also have a comparison to a short storage time (1 h) as well as no storage. While the samples stored at fridge temperature over a 96 h period still yielded reliable differential blood counts, the evaluation process was often found to be time-consuming and difficult due to the changes that can occur in the blood cells and exacerbate an easy differentiation of the leucocytes. Therefore, based on results of this investigation, samples should not be stored for over 48 h, independent of the storage temperature.

If blood samples must be stored for over 48 h, they should be placed in the fridge, as changes in the differential blood count began to occur after 48 h storage at room temperature. Samples stored at room temperature for 48 h or more will most likely not procure reliable results and should be discarded. When shipping of blood samples to an external laboratory for evaluation of the differential blood count is necessary, problems may arise during warmer months. Express delivery is inevitable and the samples should

be packed in isolating material. Additional cooling should be provided in hot climates or during warm seasons, for example with ice packs. Alternatively, fixed smears instead of blood samples should be shipped to the laboratory for staining and evaluation, to avoid a negative influence of the temperature and shipping time altogether. Fixation can be performed by submerging the dried smears in methanol ($\geq$ 99,5 %) for 10 minutes.

6. Summary

In the presented study the influence of the storage time and storage temperature of blood samples on the interpretability and results in blood smears from Hermann's tortoises *(Testudo hermanni)* was investigated, with emphasis on the differential blood count and the morphological changes in the blood cells during storage. To rule out influences from individual animals but gather a larger amount of results, the same animals were sampled repeatedly once a month. From each blood sample, containing lithium heparin as anticoagulans, smears were produced directly after the blood sampling and subsequently, aliquots were stored at room temperature or fridge temperature. After 1 h, 48 h and 96 h, smears were produced from each aliquot and stained with a Pappenheim stain. The smears were then evaluated according to a standardised protocol, including an assessment of predefined criteria for macroscopic and microscopic quality as well as the bacterial growth and the cytoplasmic vacuoles in the erythrocytes. A differential blood count was established as well. During the microscopic evaluations, a photographic documentation of the blood cells was performed, including the changes occuring after prolonged storage.

While the macroscopic quality did not show a strong influence of storage time and temperature, the microscopic quality mostly declined with prolonged storage in both temperatures. The bacterial growth and the cytoplasmic vacuoles in erythrocytes both increased distinctly with prolonged storage at room temperature. For the fridge temperature, the increase was less distinct, but existent. The leucocyte percentage values showed a clear difference between the two storage temperatures. While the percentage distribution remained uninfluenced by fridge temperature, the specimens stored at room temperature yielded a decline of heterophils and an increase of lymphocytes with prolonged storage. When investigating the morphological changes, changes in cell size and colour were found

as well as lysed cells and cytoplasmic vacuoles. In some smears produced from blood with prolonged storage it became difficult to distinguish between heterophils and eosinophils. The differentiation of thrombocytes and small lymphocytes was difficult to start with and this problem increased with prolonged storage. This investigation also revealed an influence of the month of blood sampling and the individual animals on the quality parameters of the smears.

Furthermore, a photographic reference for the blood cells of *T. hermanni* is provided, showing the blood cells in their normal, unaffected appearance as well as an exemplary presentation of the morphological changes. In this investigation the existence of two types of thrombocytes was documented for the first time for *T. hermanni*. These photographs may serve as a reference for practitioners, to correctly identify the different cell types in *T. hermanni* and to avoid the mistaking of storage artefacts as pathological signs.

From the results, it can be concluded that blood smears should be stored at fridge temperature for a maximum of 48 h before producing smears to obtain reliable results. Blood samples stored at fridge temperature for 96 h still yielded a reliable differential blood count, but were much more tedious to evaluate. Blood samples stored at room temperature for 48 h and more did not procure reliable results and should be discarded. If it is necessary to store blood samples for durations of 48 h or more, for example when shipping to external laboratories, appropriate measures are recommended, such as isolated packaging and additional cooling with ice packs or similar in hot climates.

This study was aimed at providing recommendations for the handling of blood smears in veterinary practice to contribute towards the improved medical care of reptiles. In view of the current debate about the captive husbandry of reptiles in private ownership with a consequential possible initiation of positive or negative lists in Germany, this study represents fundamental research which is still necessary to ensure medical care for reptiles based on a scientifically well-grounded knowledge.

7. Zusammenfassung

Einfluss von Lagerungsdauer und -temperatur von Blutproben auf die Beurteilung von Blutausstrichen bei Griechischen Landschildkröten *(Testudo hermanni)*

Die vorliegende Studie untersuchte den Einfluss der Lagerungsdauer und der Lagerungstemperatur von Blutproben auf die Auswertbarkeit und die Ergebnisse von Blutausstrichen bei Griechischen Landschildkröten *(Testudo hermanni)*. Dabei lag der Schwerpunkt auf dem Differentialblutbild und den morphologischen Veränderungen der Blutzellen. Um eine größere Probenanzahl zu erhalten, aber eine Beeinflussung der Ergebnisse durch individuelle Abweichungen innerhalb der Tiere möglichst auszuschließen, wurden dieselben Tiere mehrfach beprobt. Von jeder Blutprobe, mit Lithium-Heparin als Antikoagulans, wurden Ausstriche direkt nach der Blutentnahme angefertigt. Anschließend wurden die Blutproben zu gleichen Teilen jeweils bei Kühlschrank- und Raumtemperatur gelagert. Nach 1 h, 48 h und 96 h wurden von jeder Teilprobe Ausstriche angefertigt. Alle Ausstriche wurden mit einer Pappenheim-Färbung angefärbt und anschließend nach einem standardisierten Protokoll ausgewertet. Die Auswertung beinhaltete die Bewertung von makroskopischer und mikroskopischer Qualität anhand von vordefinierten Kriterien sowie die Bewertung des Bakterienwachstums und der zytoplasmatischen Vakuolen in den Erythrozyten. Ein Differentialblutbild wurde ebenfalls erstellt.

Während die makroskopische Qualität durch die Lagerungsdauer und die Lagerungstemperatur nicht stark beeinflusst wurde, verschlechterte sich die mikroskopische Qualität mit zunehmender Lagerungsdauer bei beiden Temperaturen. Das Bakterienwachstum und

die zytoplasmatischen Vakuolen in Erythrozyten zeigten mit zunehmender Lagerungsdauer bei Raumtemperatur einen deutlichen Anstieg. Bei Kühlschranktemperatur war dieser Anstieg ebenfalls vorhanden, jedoch weniger ausgeprägt. Die Leukozytenwerte wiesen einen deutlichen Unterschied zwischen den beiden Lagerungstemperaturen auf. Während die prozentuale Verteilung durch die Lagerung bei Kühlschranktemperatur unbeeinflusst blieb, zeigte sich bei Raumtemperatur mit zunehmender Lagerungsdauer ein Abfall der Heterophilen und ein Anstieg der Lymphozyten. Bei der Untersuchung der morphologischen Veränderungen wurden Veränderungen der Zellgröße und -farbe ebenso gefunden wie lysierte Zellen und zytoplasmatische Vakuolen. In einigen Ausstrichen von Blutproben mit verlängerter Lagerungsdauer wurde es schwierig, zwischen Heterophilen und Eosinophilen zu unterscheiden. Die Unterscheidung von Thrombozyten und kleinen Lymphozyten war von Anfang an schwierig, was sich mit zunehmender Lagerungszeit weiter verschlechterte. Weiterhin zeigte sich in dieser Untersuchung ein Einfluss des Monats der Blutabnahme und des Einzeltiers auf die Qualitätsparameter des Ausstriches.

Während der mikroskopischen Auswertungen wurde eine fotografische Referenz der Blutzellen von *T. hermanni* erstellt. Diese zeigt das normale, unbeeinflusste Erscheinungsbild der Blutzellen, sowie eine beispielhafte Dokumentation der morphologischen Veränderungen durch verlängerte Lagerung. In dieser Studie wurden erstmalig zwei verschiedene Typen von Thrombozyten bei *T. hermanni* dokumentiert. Diese Fotografien können als Referenz für Praktiker dienen, um die verschiedenen Zelltypen bei *T. hermanni* korrekt zu identifizieren und außerdem eine Einstufung von Lagerungsartefakten als pathologische Anzeichen zu vermeiden.

Anhand der Ergebnisse kann darauf geschlossen werden, dass, um verlässliche Ergebnisse zu erhalten, Blutproben bei Kühlschranktemperatur für maximal 48 h gelagert werden sollten. Die Blutproben lieferten nach Lagerung für 96 h bei Kühlschranktemperatur zwar nach wie vor verlässliche Ergebnisse, allerdings war die Auswertung zeitaufwendig und mühselig. Blutproben, die bei Raumtemperatur für 48 h und mehr gelagert wurden, ergaben keine verlässlichen Ergebnisse mehr und sollten verworfen werden. Falls eine Aufbewahrung von Blutproben für 48 h oder mehr notwendig ist, beispielsweise bei Versand zu externen Laboren, werden entsprechende Maßnahmen empfohlen, wie isolierende

Verpackungen und zusätzliche Kühlung mittels Kühlakkus oder ähnlichem bei warmem Wetter.

Diese Studie zielte darauf ab, Empfehlungen für den Umgang mit Blutproben in der tierärztlichen Praxis zu erstellen um einen Beitrag zur verbesserten medizinischen Versorgung von Reptilien zu leisten. Angesichts der aktuellen Diskussion um Reptilienhaltung in Privathand mit einer möglichen Einführung von Positiv- oder Negativlisten in Deutschland ist Grundlagenforschung wie die vorliegende Studie von großer Relevanz, um eine auf wissenschaftlichen Kenntnissen basierende medizinische Versorgung von Reptilien sicherzustellen.

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Appendices

A. Evaluation of blood smears - Results

The Table A.1 presents the collected data from the evaluation of the blood smears. The specifications are as follows:

Stor. temp. = Storage temperature of the blood sample

- 0 = smear produced directly after blood sampling
- 1 = storage at fridge temperature
- 2 = storage at room temperature

Stor. time = Storage time of the blood sample

- 1 = smear produced directly after blood sampling
- 2 = storage for 1 h
- 3 = storage for 48 h
- 4 = storage for 96 h

Mac. qual. = Macroscopic quality of the blood smear, the rating is as follows:

- 1 = good
- 2 = sufficient
- 3 = unsatisfactory

Mic. qual. = Microscopic quality of the blood smear, the rating is as follows:

- 1 = good
- 2 = sufficient
- 3 = unsatisfactory

Bact. growth = Bacterial growth in the blood smear, the rating is as follows:

- 0 = none
- 1 = sparse
- 2 = medium
- 3 = plenty

C. v. in ery. = Cytoplasmic vacuoles in erythrocytes in the blood smear, the rating is as follows:

- 0 = none
- 1 = sparse
- 2 = medium
- 3 = plenty

Het. = Heterophil count of 100 leucocytes.

Eos. = Eosinophil count of 100 leucocytes.

Bas. = Basophil count of 100 leucocytes.

Lymp. = Lymphocyte count of 100 leucocytes.

Mono. = Monocyte count of 100 leucocytes.

Thromb. = Thrombocyte count during the differentiation of 100 leucocytes.

Table A.1. Results from the first evaluation of the 210 blood smears.

Smear #	Animal #	Month	Stor. temp.	Stor. time	Mac. qual.	Mic. qual.	Bact. growth	C. v. in ery.	Het.	Eos.	Bas.	Lymp.	Mono.	Plasma cells	Thromb.
1	1	7	0	1	2	1	0	0	24	23	0	53	0	0	92
2	1	7	0	1	2	1	0	0	30	15	1	54	0	0	76
3	1	7	0	1	2	3	0	0	26	15	4	55	0	0	108
4	1	7	1	2	1	3	0	0	31	29	0	40	0	0	49
5	1	7	1	2	2	3	0	0	29	32	1	38	0	0	44
6	1	7	1	2	2	3	0	0	30	18	1	51	0	0	58
7	1	7	2	2	1	2	0	0	26	16	2	52	4	0	94
8	1	7	2	2	2	1	0	0	25	18	1	55	1	0	80
9	1	7	2	2	1	2	0	0	26	22	0	52	0	0	56
10	1	7	1	3	1	3	0	0	19	22	2	57	0	0	103
11	1	7	1	3	1	3	0	0	25	27	1	46	1	0	118
12	1	7	1	3	2	2	0	0	21	26	1	51	1	0	101
13	1	7	2	3	1	2	0	1	30	25	0	44	1	0	67
14	1	7	2	3	1	2	0	1	32	18	0	49	1	0	63
15	1	7	2	3	1	2	0	1	33	15	2	50	0	0	65
16	1	7	1	4	2	2	0	0	27	15	2	54	2	0	79
17	1	7	1	4	1	2	0	1	23	22	0	55	0	0	86
18	1	7	1	4	1	2	0	0	29	11	1	55	4	0	91
19	1	7	2	4	2	3	1	2	3	12	3	82	0	0	143
20	1	7	2	4	1	3	2	1	8	9	2	79	2	0	139
21	1	7	2	4	2	3	2	3	5	10	0	84	1	0	152
22	2	7	0	1	2	2	0	0	31	3	0	65	1	0	139
23	2	7	0	1	2	3	0	0	24	5	1	69	1	0	107
24	2	7	0	1	1	1	0	0	27	4	1	66	2	0	113
25	2	7	1	2	1	1	0	0	32	5	0	59	4	0	107

Table A.1 – continued from previous page

Smear #	Animal #	Month	Stor. temp.	Stor. time	Mac. qual.	Mic. qual.	Bact. growth	C. v. in ery.	Het.	Eos.	Bas.	Lymp.	Mono.	Plasma cells	Thromb.
26	2	7	1	2	1	2	0	0	30	4	1	61	4	0	99
27	2	7	1	2	2	3	0	0	33	3	0	64	0	0	93
28	2	7	2	2	1	2	0	0	29	4	3	61	3	0	103
29	2	7	2	2	1	1	0	0	31	5	1	63	0	0	114
30	2	7	2	2	2	2	0	0	28	4	1	65	2	0	98
31	2	7	1	3	2	3	0	0	30	2	1	64	3	0	132
32	2	7	1	3	2	3	0	0	32	3	1	61	3	0	119
33	2	7	1	3	2	3	0	0	29	7	1	57	6	0	106
34	2	7	2	3	1	3	0	1	37	6	2	54	1	0	116
35	2	7	2	3	2	2	0	1	34	6	0	59	1	0	108
36	2	7	2	3	2	3	0	1	29	6	0	62	3	0	122
37	2	7	1	4	2	3	0	3	18	10	0	71	1	0	171
38	2	7	1	4	2	2	0	3	24	4	1	70	2	0	102
39	2	7	1	4	1	3	0	3	13	4	1	81	1	0	148
40	2	7	2	4	2	3	3	2	2	1	0	97	0	0	110
41	2	7	2	4	2	2	3	3	2	0	0	98	0	0	52
42	2	7	2	4	2	3	3	2	3	2	0	95	0	0	49
43	3	7	0	1	1	2	0	0	30	10	2	57	1	0	102
44	3	7	0	1	1	1	0	0	27	14	0	59	0	0	88
45	3	7	0	1	1	1	0	0	19	18	0	62	1	0	92
46	3	7	1	2	2	1	0	0	19	21	1	59	0	0	91
47	3	7	1	2	2	1	0	0	22	14	3	61	0	0	96
48	3	7	1	2	3	2	0	0	23	17	0	59	1	0	75
49	3	7	2	2	2	3	0	0	28	17	0	53	2	0	96
50	3	7	2	2	2	1	0	0	27	21	0	51	1	0	85
51	3	7	2	2	2	1	0	0	25	19	3	52	1	0	71

Table A.1 – continued from previous page

Smear #	Animal #	Month	Stor. temp.	Stor. time	Mac. qual.	Mic. qual.	Bact. growth	C. v. in ery.	Het.	Eos.	Bas.	Lymp.	Mono.	Plasma cells	Thromb.
52	3	7	1	3	1	3	0	0	23	13	3	59	2	0	109
53	3	7	1	3	3	3	0	0	21	7	1	70	1	0	86
54	3	7	1	3	2	3	0	0	8	26	5	58	3	0	109
55	3	7	2	3	3	2	0	1	12	16	1	70	1	0	86
56	3	7	2	3	3	3	0	0	16	24	2	57	0	0	60
57	3	7	2	3	3	3	0	1	11	24	3	62	0	0	99
58	3	7	1	4	2	3	0	0	23	14	1	59	3	0	88
59	3	7	1	4	2	2	0	1	20	16	1	58	5	0	100
60	3	7	1	4	2	2	0	0	17	17	1	62	3	0	117
61	3	7	2	4	1	3	0	0	3	24	0	73	0	0	63
62	3	7	2	4	2	3	2	1	0	24	0	75	1	0	80
63	3	7	2	4	1	2	1	1	0	29	0	69	2	0	62
64	4	7	0	1	1	2	0	0	55	7	2	35	1	0	131
65	4	7	0	1	2	2	0	0	40	14	5	41	0	0	177
66	4	7	0	1	2	1	0	0	49	9	3	39	0	0	148
67	4	7	1	2	2	3	0	0	26	12	2	59	1	0	62
68	4	7	1	2	2	2	0	0	50	15	0	35	0	0	25
69	4	7	1	2	2	2	0	0	45	12	2	41	0	0	59
70	4	7	2	2	1	1	0	0	51	16	2	31	0	0	27
71	4	7	2	2	2	1	0	0	50	14	1	35	0	0	21
72	4	7	2	2	2	2	0	0	54	12	0	34	0	0	13
73	4	7	1	3	3	1	0	0	48	12	3	36	1	0	61
74	4	7	1	3	2	2	0	0	48	16	0	36	0	0	57
75	4	7	1	3	3	2	0	0	54	11	4	31	0	0	60
76	4	7	2	3	3	2	0	0	51	11	3	35	0	0	56
77	4	7	2	3	2	1	0	0	55	9	3	32	1	0	66

Table A.1 – continued from previous page

Smear #	Animal #	Month	Stor. temp.	Stor. time	Mac. qual.	Mic. qual.	Bact. growth	C. v. in ery.	Het.	Eos.	Bas.	Lymp.	Mono.	Plasma cells	Thromb.
78	4	7	2	3	2	2	0	0	46	12	2	25	15	0	51
79	4	7	1	4	2	2	0	3	51	15	1	34	0	0	107
80	4	7	1	4	2	3	0	3	40	14	3	43	0	0	117
81	4	7	1	4	2	3	0	0	35	13	1	48	3	0	114
82	4	7	2	4	2	3	1	2	5	2	1	92	0	0	116
83	4	7	2	4	2	2	2	1	13	23	2	62	0	0	84
84	4	7	2	4	2	3	1	2	8	14	2	76	0	0	89
85	1	8	0	1	2	1	0	0	34	9	2	53	0	0	47
86	1	8	0	1	2	1	0	0	27	14	4	54	1	0	60
87	1	8	0	1	1	1	0	0	27	9	2	62	0	0	60
88	1	8	1	2	1	1	0	0	36	14	3	47	0	0	16
89	1	8	1	2	2	2	0	0	35	11	1	53	0	0	42
90	1	8	1	2	2	2	0	0	30	20	2	48	0	0	25
91	1	8	2	2	2	2	0	0	28	12	1	58	1	0	51
92	1	8	2	2	2	2	0	0	31	21	2	45	1	0	65
93	1	8	2	2	2	1	0	0	30	18	4	48	0	0	68
94	1	8	1	3	1	3	1	0	21	22	0	54	3	0	107
95	1	8	1	3	2	3	0	0	19	27	3	49	2	0	101
96	1	8	1	3	2	2	0	0	19	21	0	59	1	0	98
97	1	8	2	3	1	2	0	1	2	31	0	66	1	0	88
98	1	8	2	3	1	3	0	1	4	26	0	69	1	0	103
99	1	8	2	3	1	3	0	0	2	29	0	67	2	0	102
100	1	8	1	4	1	3	0	1	27	21	3	46	3	0	39
101	1	8	1	4	2	2	0	1	21	22	3	53	1	0	92
102	1	8	1	4	2	2	0	1	24	15	5	54	2	0	97
103	1	8	2	4	2	3	1	2	1	28	3	67	1	0	119

Table A.1 – continued from previous page

Smear #	Animal #	Month	Stor. temp.	Stor. time	Mac. qual.	Mic. qual.	Bact. growth	C. v. in ery.	Het.	Eos.	Bas.	Lymp.	Mono.	Plasma cells	Thromb.
104	1	8	2	4	1	3	1	3	0	22	4	73	1	0	107
105	1	8	2	4	2	3	1	3	1	25	2	70	2	0	136
106	2	8	0	1	1	1	0	0	23	21	0	56	0	0	94
107	2	8	0	1	1	1	0	0	18	14	1	67	0	0	103
108	2	8	0	1	1	1	0	0	19	24	0	56	1	0	98
109	2	8	1	2	2	1	0	0	12	18	2	68	0	0	117
110	2	8	1	2	2	1	0	0	8	25	2	63	2	0	96
111	2	8	1	2	2	2	0	0	22	24	2	52	0	0	119
112	2	8	2	2	2	1	0	0	26	26	2	46	0	0	103
113	2	8	2	2	1	2	0	0	26	21	1	52	0	0	117
114	2	8	2	2	2	1	0	0	17	29	1	52	1	0	86
115	2	8	1	3	2	2	0	0	17	30	0	52	1	0	76
116	2	8	1	3	2	2	0	0	10	32	1	55	2	0	98
117	2	8	1	3	2	2	0	0	17	27	1	53	2	0	97
118	2	8	2	3	2	3	1	1	8	37	2	53	0	0	87
119	2	8	2	3	1	2	1	2	13	32	1	54	0	0	119
120	2	8	2	3	1	3	2	2	13	36	3	48	0	0	96
121	2	8	1	4	1	3	0	1	15	34	4	46	1	0	104
122	2	8	1	4	2	2	0	1	8	28	4	58	2	0	93
123	2	8	1	4	2	2	0	1	8	31	1	59	1	0	118
124	2	8	2	4	3	2	3	2	3	37	4	56	0	0	82
125	2	8	2	4	2	3	3	2	2	9	3	86	0	0	106
126	2	8	2	4	3	3	3	2	0	18	3	79	0	0	93
127	1	9	0	1	2	1	0	0	30	10	1	57	2	0	92
128	1	9	0	1	2	1	0	0	27	8	2	62	1	0	67
129	1	9	0	1	2	1	0	0	37	6	1	55	1	0	73

Table A.1 – continued from previous page

Smear #	Animal #	Month	Stor. temp.	Stor. time	Mac. qual.	Mic. qual.	Bact. growth	C. v. in ery.	Het.	Eos.	Bas.	Lymp.	Mono.	Plasma cells	Thromb.
130	1	9	1	2	1	1	0	0	35	8	4	53	1	0	77
131	1	9	1	2	2	1	0	0	25	12	1	61	1	0	102
132	1	9	1	2	2	1	0	0	30	6	2	59	3	0	68
133	1	9	2	2	1	2	0	0	31	4	3	59	3	0	128
134	1	9	2	2	1	1	0	0	37	8	0	55	0	0	127
135	1	9	2	2	1	2	0	0	27	9	2	62	0	0	137
136	1	9	1	3	2	2	0	0	12	9	3	74	2	0	154
137	1	9	1	3	2	1	0	0	20	5	3	72	0	0	89
138	1	9	1	3	2	1	0	0	12	7	1	79	1	0	120
139	1	9	2	3	1	1	0	0	20	9	4	66	1	0	121
140	1	9	2	3	1	2	0	0	23	6	0	70	1	0	98
141	1	9	2	3	1	1	0	0	19	12	0	68	1	0	101
142	1	9	1	4	2	1	0	1	13	5	4	77	1	0	103
143	1	9	1	4	2	1	0	2	23	7	4	64	2	0	114
144	1	9	1	4	2	1	0	2	17	6	6	70	1	0	108
145	1	9	2	4	2	3	3	2	5	5	2	88	0	0	120
146	1	9	2	4	2	3	3	2	3	5	0	91	1	0	107
147	1	9	2	4	2	3	3	1	2	3	0	95	0	0	137
148	2	9	0	1	2	3	0	0	31	12	0	56	1	0	72
149	2	9	0	1	1	2	0	0	21	12	0	65	2	0	144
150	2	9	0	1	2	2	0	0	21	9	1	68	1	0	128
151	2	9	1	2	1	2	0	0	3	11	1	85	0	0	137
152	2	9	1	2	1	2	0	0	15	10	1	73	1	0	132
153	2	9	1	2	1	2	0	0	25	8	1	66	0	0	132
154	2	9	2	2	1	3	0	0	29	20	1	48	2	0	134
155	2	9	2	2	1	3	0	0	19	11	2	67	1	0	151

Table A.1 – continued from previous page

Smear #	Animal #	Month	Stor. temp.	Stor. time	Mac. qual.	Mic. qual.	Bact. growth	C. v. in ery.	Het.	Eos.	Bas.	Lymp.	Mono.	Plasma cells	Thromb.
156	2	9	2	2	1	2	0	0	35	18	0	47	0	0	89
157	2	9	1	3	2	3	0	0	13	31	1	55	0	0	173
158	2	9	1	3	2	3	0	0	16	36	0	48	0	0	118
159	2	9	1	3	2	3	0	0	18	40	0	41	1	0	142
160	2	9	2	3	2	3	2	1	5	39	0	55	1	0	143
161	2	9	2	3	2	3	1	1	2	34	0	62	2	0	121
162	2	9	2	3	1	3	2	1	4	39	1	55	1	0	137
163	2	9	1	4	2	3	1	0	12	31	0	55	2	0	41
164	2	9	1	4	2	2	1	0	28	27	0	43	2	0	45
165	2	9	1	4	2	2	1	0	15	42	0	42	1	0	49
166	2	9	2	4	2	3	3	3	2	8	0	57	0	0	58
167	2	9	2	4	1	3	3	3	0	16	0	7	0	0	112
168	2	9	2	4	1	3	3	3	0	7	0	10	0	0	109
169	1	10	0	1	1	1	0	0	22	4	4	68	2	0	120
170	1	10	0	1	1	2	0	0	30	6	3	60	1	0	103
171	1	10	0	1	1	1	0	0	21	5	4	69	1	0	104
172	1	10	1	2	3	2	0	0	25	5	0	69	1	0	41
173	1	10	1	2	2	3	0	0	25	6	3	65	1	0	56
174	1	10	1	2	2	2	0	0	41	5	4	50	0	0	82
175	1	10	2	2	2	2	0	0	18	7	7	67	1	0	134
176	1	10	2	2	2	1	0	0	31	2	8	56	3	0	112
177	1	10	2	2	2	1	0	0	29	4	5	62	0	0	147
178	1	10	1	3	2	3	0	0	36	13	0	50	1	0	58
179	1	10	1	3	2	2	0	0	25	5	2	68	0	0	134
180	1	10	1	3	1	2	0	1	40	7	0	53	0	0	57
181	1	10	2	3	1	2	0	0	22	11	1	66	0	0	147

Table A.1 – continued from previous page

Smear #	Animal #	Month	Stor. temp.	Stor. time	Mac. qual.	Mic. qual.	Bact. growth	C. v. in ery.	Het.	Eos.	Bas.	Lymp.	Mono.	Plasma cells	Thromb.
182	1	10	2	3	2	2	0	0	29	4	2	63	2	0	145
183	1	10	2	3	1	2	0	0	24	7	0	68	1	0	147
184	1	10	1	4	2	2	0	2	20	7	2	68	3	0	135
185	1	10	1	4	2	2	0	2	30	8	0	62	0	0	144
186	1	10	1	4	2	2	0	0	27	6	2	64	1	0	146
187	1	10	2	4	2	2	3	2	6	3	9	81	1	0	168
188	1	10	2	4	2	2	3	2	5	1	11	80	3	0	138
189	1	10	2	4	2	2	3	2	10	1	9	78	2	0	164
190	2	10	0	1	2	2	0	0	32	5	0	61	2	0	152
191	2	10	0	1	2	2	0	0	34	2	1	61	2	0	137
192	2	10	0	1	2	1	0	0	41	4	2	53	0	0	134
193	2	10	1	2	2	1	0	0	27	8	1	64	0	0	132
194	2	10	1	2	1	1	0	0	22	4	0	73	1	0	131
195	2	10	1	2	2	2	0	0	29	4	0	65	2	0	161
196	2	10	2	2	2	3	0	0	35	4	0	60	1	0	140
197	2	10	2	2	1	3	0	0	42	5	0	53	0	0	117
198	2	10	2	2	2	1	0	0	40	8	0	52	0	0	69
199	2	10	1	3	3	2	0	0	25	13	0	61	1	0	101
200	2	10	1	3	2	1	0	0	25	9	2	63	1	0	117
201	2	10	1	3	1	2	0	0	29	8	1	62	0	0	104
202	2	10	2	3	1	3	1	1	9	20	0	68	3	0	148
203	2	10	2	3	1	3	0	0	13	19	0	68	0	0	148
204	2	10	2	3	1	3	0	0	14	13	1	70	2	0	143
205	2	10	1	4	3	2	0	2	20	6	4	67	3	0	93
206	2	10	1	4	2	2	0	2	21	9	2	68	0	0	136
207	2	10	1	4	2	2	0	2	33	9	3	53	2	0	134

Table A.1 – continued from previous page

Smear #	Animal #	Month	Stor. temp.	Stor. time	Mac. qual.	Mic. qual.	Bact. growth	C. v. in ery.	Het.	Eos.	Bas.	Lymp.	Mono.	Plasma cells	Thromb.
208	2	10	2	4	2	3	1	3	14	28	1	55	2	0	146
209	2	10	2	4	2	2	0	3	9	18	2	71	0	0	163
210	2	10	2	4	1	2	0	2	19	29	3	49	0	0	141

B. Protocols

B.1. Health monitoring of the tortoises

The following protocols were designed and used for the extensive health check of the tortoises before the beginning of the study and the clinical examination performed before each blood sampling.

Health check before beginning of the study Date: _____

Species: *Testudo hermanni*	Animal ID:
Size in cm (Shell length):	Age (if known):
Particular characteristics:	Sex:

Clinical examination:	see Protocol: Clinical examination
Blood examination (abnormalities):	
X-ray (dv, ll and cc):	
Faeces examination:	

Protocol for the clinical examination

Date: _____ Animal ID: _____

Body weight:	BCS (according to protocol):
Observation (assess first without handling): O Species-specific: - shy, withdraws into shell - calm - alert, mobile - hissing O Deviation:	Integument: O Species-specific: - Shell: hard and unrelenting (harder with increasing age), regular structure, narrow bone seams - Skin: leathery, dry, signs of ecdysis may be present and species-specific covered with horny scales (Head, extremities, tail) O Deviation:
Mouth: O Species-specific: -mucous menbranes pale pink, moist, no coverings or general discoloration - Beak species-specific long and hard O Deviation:	Nares: O Symmetric, clear, dry O Deviation:
Eyes: O Symmetric, open or not permanently closed, not retracted, no exopthalmus, no discharge, cornea and lens clear O Deviation:	Ears: O Dry, no swelling O Deviation:
Extremities: O Symmetric, no swellings, well muscled, claws present with species-specific length O Deviation:	Cloaca: O Dry, symmetric shape, no adhesions, but faeces remnants may be present O Deviation:
Palpation of the coelomic cavity: O Intestinal loops may be palpable, possibly the bladder with high filling O Deviation:	

B.2. Evaluation of blood smears

The following protocol was designed and modified according to Corrons et al. [2004]; Kraft et al. [2005] and Pendl et al. [2008].

EVALUATION OF BLOOD SMEARS

Specifications:

Smear Nr.:	Evaluation date:
Animal Nr.:	Species: *Testudo hermanni*
Storage time:	Storage temperature:

Macroscopic evaluation

Tick boxes, then summarize accordingly as quality.

Criteria	**Yes**	**No**
Smear is 3-4 cm long, at least 2,5 cm		
Pink in thin part and purplish-blue in thicker parts		
Smear becomes thinner towards the end, showing a “tongue”		
Not too thin or too thick		
Not staged, “tremulous”		
Not showing holes		

→ Macroscopic quality:

O Good (5-6 x Yes)
O Sufficient (3-4 x Yes)
O Unsatisfactory (0-2 x Yes)

Comments/other findings (both macroscopic and microscopic)

Microscopic evaluation and differential blood count

Leucocytes						Thrombocytes	Smear Nr.:		
Granulocytes			Mononucleated leucocytes				**Microscopic evaluation**		
Acidophilic		Basophilic					Tick boxes, then summarize accordingly as quality.		
Heterophils	Eosinophils	Basophils	Lymphocytes	Monocytes	Plasma cells		**Criteria**	**Yes**	**No**
							Cells spread out evenly		
							No or minimal precipitation		
							Uniform staining over slide		
							→ Microscopic quality:		
							O Good (3 x Yes) O Sufficient (2 x Yes) O Unsatisfactory (0-1 x Yes)		
							For the following criteria, use a rating system ranging from – (none), + (sparse, 1-5 per field of view) and ++ (medium, 6-10 per field of view) to +++ (plenty, > 11 per field of view)		
							Bacterial growth		
							Cytoplasmic vacuoles in erythrocytes		
							Comments:		
Σ/%						Σ			

Microscope: Leitz Aristoplan, objective lens 100x, ocular 10x, camera: Leica WILD MPS 52V

Examine blood smear in a meandering course, from margin to middle to margin, using immersion oil. Count and differentiate a total of 100 leucocytes for differential blood count, using a counter and enter data accordingly in boxes.

C. Chemical blood values of the tortoises

The following table (Table C.1) shows the chemical blood values ascertained in the context of the health check that was performed before the beginning of the study to ensure the health of all tortoises involved.

Table C.1. The chemical blood values of the tortoises from the health check performed before the beginning of the study.

	Unit	*T. hermanni* #1	*T. hermanni* #2	*T. hermanni* #3	*T. hermanni* #4
ALP	U/l	117	302	152	158
AST	U/l	55	92	63	66
ALAT	U/l	4	9	3	14
LDH	U/l	100	220	134	54
CK	U/l	27	655	29	90
Triglycerides	mmol/l	0.49	0.21	0.68	0.55
BUN	mmol/l	<0.83	<0.83	<0.83	0.67
UA	μmol/l	137	155	250	107
Na	mmol/l	139	134	125	137
K	mmol/l	3.6	4.6	3.8	3.9
Ca	mmol/l	2.33	2.5	2.07	2.32
P	mmol/l	0.78	1.07	0.61	0.78
Glucose	mmol/l	3.05	4.16	2.44	3.33
Albumin	g/l	6.3	11.7	5.6	7.3
TP	g/l	23	36	20	29

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